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ROLE OF MATRIX METALLOPROTEINASE-2 IN THEROSCLEROSIS AND ABDOMINAL AORTIC ANEURYSMS IN APOLIPOPROTEIN E DEFICIENT MICE

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ABSTRACT OF DISSERTATION

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ABSTRACT OF DISSERTATION

A dissertation submitted in partial fulfillment of the
requirements for the degree of Doctor of Philosophy in the
College of Medicine
at the University of Kentucky

By
Jing Huang
Lexington, Kentucky

Director: Dr. Alan Daugherty, Professor of Medicine and Physiology

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Matrix metalloproteinase-2 (MMP-2, gelatinase A, type IV collagenase) is a member of a family of zinc-dependent metalloendopeptidases that functions in the degradation of elastin, collagens, and other components of extracellular matrix (ECM). Both secretion and activation of MMP-2 are elevated in human atherosclerotic lesions and abdominal aortic aneurysms (AAA). In this dissertation project, we sought to test the hypothesis that MMP-2 plays a critical role in both atherosclerosis and AAA. We also sought to determine the detailed mechanism. We first examined the atherosclerosis and AngII-induced AAAs development in MMP-2-/- x apolipoprotein (apoE)-/- mice in vivo. It was surprising that MMP-2 deficiency did not reduce the incidence of AngII-induced AAAs or the size of atherosclerosis in apoE-/- mice. However, the cellular and ECM content of atherosclerotic plaques were modified in MMP-2-/- x apoE-/- mice as compared to MMP-2+/+ x apoE-/- control mice. To explain the apparent paradox between this result and the hypothesis, we investigated the morphological characteristics of the aortic wall of MMP-2-/- mice. We detected an

enhanced MMP-9 level in the aortic wall of MMP-2-/- x apoE-/- mice compared with MMP-2+/+ x apoE-/- mice. Interestingly, we also observed more branching of the elastin fibers in aortic wall of MMP-2-/- mice as compared with aorta of wild type mice. We also examined the behavior of macrophages from MMP-2-/- mice. Reduced adhesion, migration, and expression of integrin beta 3 were detected in MMP-2 deficient macrophages compared with wild type macrophages. Lastly, we examined whether MMP-2 deficiency in bone marrow-derived cells may influence AAAs and atherosclerosis using bone marrow transplantation technique. There was a significant reduction of both atherosclerosis development and AAAs formation in mice that were reconstituted MMP-2-/- bone marrow cells. In conclusion, the findings in this dissertation suggest that MMP-2 might play an important role in atherosclerosis and aneurysm through influencing inflammatory cell infiltration.

KEYWORDS: matrix metalloproteinase-2, atherosclerosis, abdominal aortic aneurysm, integrin, macrophage

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Introduction

I. Atherosclerosis

Atherosclerosis

Atherosclerosis is traditionally described as a condition that involves the deposition of lipids in the tunica intima and deep layers of large and medium-sized arteries. These lipid deposits, along with other substances, form plaques which tend to diminish arterial elasticity and may result in a narrowing or blockage of the lumen in some arteries. However, the hardening of the arteries often occurs in advanced stages and is not a predominant feature of atherosclerosis. The plaque formation constricts the blood flow and ultimately deprives the blood supply to vital organs. Based on an official survey, coronary heart disease (CHD), the most common form of heart disease, was the leading cause of death (2,443,387 deaths; 241.7/population of 100,000) in the USA for 2002.[112] The same survey indicated that stroke, which is another major complication of atherosclerosis, was the third leading cause of death accounting for 162,672 deaths (56.4/population of 100,000).[112]

Pathogenesis

According to many epidemiological reports, a series of etiologic agents including cigarette smoking, dietary fat, sex hormones, and oxidative stress are associated with the initiation and progression of atherosclerosis. Genetic factors are also important in the development of atherosclerosis. For example, carriers of the polymorphism apo (apolipoprotein) E4 alleles have a greater risk of cardiovascular diseases than individuals with the apoE3/3 genotype. Genomic instability associated with loss of heterozygosity and mutations in DNA micro

satellites have been implicated in the initiation and extension of plaques.[5] Multiple genetically predisposed diseases or conditions are closely related to atherosclerosis as well, such as some dyslipidemias, diabetes mellitus, and hypertension.

Pathological processes

Although the exact biological process of atherosclerosis is not fully understood, three different stages of atherosclerosis have been described – the "fatty streak" stage, the fibrous plaque stage, and the complicated lesion stage. The appearance of a viscous, yellow streak running along the endothelial surface of the arteries is the earliest pathological stage of atherosclerosis. It is the result of accumulation of serum lipoproteins within the intima of the vessel wall. Microscopy reveals that varying proportions of lipid-laden foam cells (monocyte-derived macrophages), T lymphocytes, and smooth muscle cells constitute these "streaks." The lesions are focal, small, and non-obstructive. As the disease advances into the second stage, the fatty streak may progress to form a fibrous plaque, which is the result of progressive lipid accumulation and the migration and proliferation of smooth muscle cells. These smooth muscle cells are responsible for the deposition of extracellular matrix (ECM) components and formation of a fibrous cap that overlies a core of lipid-laden foam cells, extracellular lipid, and necrotic cellular debris. Growth of the fibrous plaque results in vascular remodeling, progressive luminal narrowing, blood-flow abnormalities, and usually compromises oxygen supply to the target organ. The last stage of atherosclerosis, the complicated lesion stage, is described as a calcified fibrous plaque containing various degrees of necrosis and ulceration, and is frequently associated with symptoms. With increasing necrosis in the fibrous plaque, the arterial wall progressively weakens. Should the plaque rupture, the cholesterol and connective tissue underneath might be exposed. Consequently, rupture provokes strong blood clotting reactions, thus arterial

emboli can be formed.

With the advancement of cardiovascular biology, the understanding of atherosclerosis has evolved rapidly over the past three decades. To date, atherosclerosis has been well characterized by endothelial dysfunction, intimal thickening, vascular smooth muscle cell (VSMC) proliferation, lipid deposition, cholesterol-laden foam cell (monocyte-derived macrophage) development, inflammatory cell accumulation, vascular matrix remodeling, excess generation of reactive oxygen species (ROS) and platelet activation and thrombosis. The balance of systemic and local inflammatory and anti-inflammatory cellular and molecular elements play a pivotal role in all phases of plaque development, progression, and degeneration.[42,138,143,170] Therefore, the infiltration and accumulation of inflammatory cells, especially macrophages, are critical events in the development of atherosclerosis. The autopsies of patients who have died of acute coronary disease reveal that macrophage infiltration and accumulation have a consistently positive relationship with plaque rupture.[55] Although the up-take of oxidatively modified LDL (ox-LDL) by macrophages and their subsequent exit from the lesion might be anti-atherogenic in the early stage of atherosclerosis, macrophages produce a diverse array of atherogenic cytokines, chemoattractants, and proteolytic enzymes including matrix metalloproteinases (MMPs), which can degrade all macromolecular constituents of the ECM in the vascular wall.

There are several hypotheses concerning the initiation of atherosclerosis. The first proposes a “response-to-injury” scenario where injury to endothelial and smooth muscle cells triggers initial events that lead to atherosclerosis. Another hypothesis, oxidation, focuses on the significance of the oxidative modification of low density lipoprotein (LDL) in subsequent atherogenic responses, such as inducing the synthesis of monocyte chemo-tactic protein-1 (MCP-1) which then induces the recruitment of inflammatory cells to lesion areas. A third proposal, “response-to-retention”, places emphasis on the subendothelial retention and accumulation of LDL and other atherogenic lipoproteins in arteries as the primary

factor for initiating cholesterol-induced atherosclerosis.[192] Intensive studies have been done to understand the molecular mechanisms underlying the progression of atherosclerosis. Many molecular pathways in the progression of atherosclerosis have been defined: 1) alterations of transforming growth factor (TGF)-beta, TGF-beta receptors, and Smad signaling proteins (their name comes from the TGF-beta homologs found in C. elegans ("Sma") and drosophila).[13,140] 2) alterations in several other peptide growth factors that regulate cell cycle and proliferation in atherosclerosis including epidermal growth factor (EGF), fibroblast growth factor (FGF), platelet-derived growth factor (PDGF), insulin-like growth factor (IGF), granulocyte macrophage colony stimulating factor (GM-CSF).[147,149,195] 3) the altered expression of cell adhesion molecules, such as integrins and selectins, which serve as promoters of atherosclerosis.[28,71] 4) the involvement of proteases and tissue protease inhibitors in the progression of atherosclerosis by contributing to luminal thrombosis.[186] 5) the deregulation of nuclear factor kappa B (NF-κB). NF-κB is a transcription factor involved in the regulation and expression of a diverse array of chemokines, cytokines, growth factors, cell adhesion molecules, and cell cycle regulatory proteins. NF-κB signaling also up-regulates the expression of pro-apoptotic proteins such as Bcl-2, and down-regulates the expression of anti-apoptotic proteins such as Bax.[9,25,34] 6) the alteration of MMP activity, which is associated with the aberrant regulation of ECM remodeling, leading to atherosclerosis.[18,67]

II. Abdominal aortic aneurysms

Abdominal aortic aneurysms

An aneurysm is defined as a localized, blood-filled, and permanent dilatation of a blood vessel caused by a disease or weakening of the vascular wall. The word aneurysm is derived from the Greek word for "widening". Connective tissue diseases such as Marfan, Ehlers-Danlos', and Stickler

syndrome as well as aging, and mycosis are associated with aneurysm formation.

Abdominal aortic aneurysm (AAA) is a chronic, degenerative disease characterized by the progressive weakening and irreversible expansion of the abdominal aortic wall. In 1991, the Society for Vascular Surgery and the International Society Cardiovascular Surgery Ad Hoc Committee on Standards in Reporting developed a criterion for AAA, stating the infra-renal abdominal aortic diameter should be more than 150% of the expected normal diameter. AAAs are prevalent in aged populations (2-9% of people >65 years of age) with rupture causing approximately 15,000 unexpected deaths every year in USA.[84] The incidence of AAA has increased dramatically in recent decades. Factors that may have contributed to this increase include the increasing mean age of the population and improvements in diagnostic evaluation. Although there is variability between patients in the rate of aortic dilation and the point of rupture, the available method of reducing associated mortality is still the detection of AAAs before rupture, followed by repair of the damaged aorta.

Pathogenesis

Although the etiology of AAAs is unknown, several population-based studies and relative hazard risk assessment evaluations indicate that the development of AAA is significantly related to multiple factors: smoking, advanced age, male gender, and a family history. There are conflicting reports concerning the association between AAA, dyslipidemia, and hypertension. [3,115,210] Traditionally, atherosclerosis is believed to contribute to the formation of AAAs; a relationship that is supported by population studies. [11,79] However, these studies could not distinguish causal effects and associated risk factors. In addition, evidence indicates that atherosclerosis is not the cause of AAAs because many patients developed severe atherosclerotic lesions in their aortas without aneurysm formation.[200] Regarding family history, although the frequency of AAAs

in first-degree relatives is much higher when compared to the unrelated population, a genetic basis for this disease remains unclear.

Pathological processes

Traditional concepts of AAAs emphasize the degenerative thickening and dilation of the aortic wall. Immunohistochemistry and molecular biology experiments with human aneurysmal tissue obtained during surgery have consistently demonstrated large numbers of inflammatory cells in the aortic wall, increased cytokine and protease activity, and elevated levels of cellular production pro-apoptosis genes (such as p53 and p21) in vascular smooth muscle cells (VSMCs).[76,148,201] MMPs and fibrinolytic enzymes are found to be more active in aneurysmal tissues than in occlusive atherosclerotic tissues. The upregulation of these proteases is believed to be responsible for the destruction of the elastin and collagen structural matrix.[21,63] Moreover, the physiological inhibitors of these proteases, such as TIMP-2 and PAI-1, have decreased expression in AAA tissue. When factors such as accumulated inflammatory cells, vascular matrix turnover by highly activated proteolytic enzymes and depletion of VSMCs are present, their combined actions weaken the structural matrix of the abdominal aortic wall, leading to aneurysmal dilation under the high transmural pulse of the aorta. Consequently, these changes are accompanied by thrombus formation in many cases.

Many studies suggest that the hallmark pathology of AAAs is the progressive and irreversible degeneration of the medial elastic lamellae. This destruction of elastic lamellae is considered to contribute to the aneurysmal dilation and enlargement. However, the vessel wall remodeling occurs by mechanisms that are different from any of the risk conditions mentioned previously.[8]

III. Matrix metalloproteinase-2 (MMP-2)

General information regarding MMPs

The matrix metalloproteinase (MMP) family is composed of over 30 endopeptideases in which at least 17 are human MMPs. These enzymes share some common features: they have the ability to cleave various macromolecules of the ECM; they are comprised of the calcium and zinc ions necessary for catalytic activity; they are produced and secreted as latent forms, except for MMP-11, 27 and MT-MMPs; and they are inhibited by their endogenous inhibitors, i.e., tissue inhibitor of matrix metalloproteinases (TIMPs). MMPs are classified into 6 subgroups based on their substrate specificity and internal homologies (Figure 1.1): collagenases (MMP-1, -8, -13, -18), gelatinases (MMP-2, -9), stromelysins (MMP-3, -10, -11, -19), matrilysins (MMP-7, -26), membrane-type MMPs (MT-MMPs: MMP-14, -15, -16, -17, -24, -25), and non-classified MMPs (MMP-12, -20, -21, -22, -23, -27, -28). They are involved in extracellular matrix degradation in many physiological and pathological processes such as wound healing, ovulation, cancer metastasis, and inflammatory joint diseases.[70,190,206] They may be implicated in cardiovascular diseases such as atherosclerosis and AAAs as well. At least 14 MMPs have been characterized in vascular cells.[147] The gelatinases (i.e. MMP-2 and MMP-9) have attracted attention for their elastolytic capabilities and increased levels in lesions of vascular diseases.

Many MMPs are highly expressed in AAAs and atherosclerotic lesions. It is believed that MMPs are involved in the destruction of the ECM and are harmful in vascular diseases such as AAAs and atherosclerosis. However, more evidence suggests that the role of MMPs is not limited in turnover of the ECM components. Actually, they have multiple substrates that are involved in the control of cellular behavior. Therefore, new evaluations of these enzymes should be performed to elucidate the underlying mechanisms in more detail. There are

several studies demonstrating direct genetic evidence of the role of MMPs and their inhibitors in vascular diseases. A few relevant conclusions are: MMP-1 transgenic mice on an apoE-/- background demonstrated decreased atherosclerotic lesions. This suggests that the remodeling of the neointimal ECM by MMP-1 is beneficial in atherosclerosis;[117] MMP-2 deficiency reduced intimal hyperplasia in a mouse carotid artery blood flow cessation model;[101,114] MMP-3 inactivation increased the size of atherosclerotic lesions and decreased the formation of aneurysm in apoE null mice;[183] MMP-9 deficiency but not MMP-12 deficiency reduced AAA formation induced by elastase perfusion in mice;[162] deficiency of MMP-9, MMP-2, and MMP-12 reduced AAA formation induced by abluminal application of calcium chloride in mice;[126,125] MMP-9 deficiency, but not MMP-12 deficiency, protected against atherosclerotic plaque growth; both deficiency of MMP-9 and 12 protected against atherosclerotic media destruction and ectasia in apoE null mice;[129] TIMP (tissue inhibitor of MMPs)-1 inactivation prevents development of atherosclerosis, but enhances aneurysm formation [182] in apoE null mice; Lemaitre et al. reported that TIMP-1 deficiency caused an increased medial degradation with pseudo-aneurysm formation in apoE-/- mice.[118]

MMP-2

The structure of the MMP-2 protein: MMP proteins have high sequence homology. Almost all of the MMP proteins contain the following domains: a signal sequence; a pro-peptide domain with a strictly conserved cystine residue which contains a Zn-ligating thiol (SH) group; a catalytic domain with a Zn binding site and a hemopexin-like C' terminal domain which contains four repeats (Figure 1.2). The only exception are the matrilysins, the smallest members of MMPs family, which do not have the hemopexin-like domain.[78] Gelatinases (MMP-2 and 9) have 3 contiguous fibronectin type II-like domains, which may contribute to specificity in substrate binding of these enzymes.[144] MMP-2, a 72

kDa protein, is called a gelatinase A or type IV collagenase because of its substrates.

MMP-2 gene expression: Unlike other MMPs, MMP-2 is primarily expressed by many cells, including VSMCs,[63] macrophages,[180, 213] fibroblasts, [154] endothelial cells,[91] myocytes,[33] mast cells,[56] and lymphocytes.[54] The promoter region of MMP-2 is unique because of the absence of the TATA and CAAT box.[89] It also lacks an upstream transforming growth factor (TGF) beta inhibitory element (TIE).[135] This suggests that MMP-2 is essentially a "housekeeping" gene. Therefore, the transcription of MMP-2 gene is rarely suppressed. For example, TGF-beta, a regulator of many MMPs, actually upregulates MMP-2 mRNA levels and increases the half-life of MMP-2 mRNA in human fibroblasts.[154] Many other cytokines can upregulate MMP-2 gene expression, especially under pathological conditions such as inflammation. For instance, granulocyte-macrophage colony-stimulating factor (GM-CSF) upregulates MMP-2 expression at both the transcription [202] and translation levels [194] in head and neck cancer cells. Interleukin (IL)-12 and IL-18 enhance MMP-2 mRNA levels separately and synergistically in monocytic cells.[1] Also, Eichler et al. and Kim et al. showed TGF-alpha enhanced MMP-2 expression in mouse blastocysts and human retinal pigment epithelial cells,[50,108] while Cipollone et al. showed that macrophage and smooth muscle cell PGE_2 increases the production of MMP-2 in the vascular wall.[32]

The activation of MMP-2: As with other members of the MMP family, MMP-2 is synthesized as a latent zymogen. MMP-2 does not undergo spontaneous activation.[110] The pro-domain is critical for keeping MMP-2 in the inactive state. The thiol group of the conserved cystine of the pro-domain binds to the catalytic zinc atom in the active site of the catalytic domain, thus keeping the enzyme inactive. The proteolytic removal of the pro-domain at Asn37-Leu38 is essential for the activation of MMP-2. After the removal of the pro-domain by chemicals or other proteases, the catalytic cleft is exposed to water to hydrolyze and disrupt the interaction of the thiol group (SH) of the conserved cysteine

residue in the pro-domain to the Zn atom.[191] Under physiological conditions, proMMP-2 is fundamentally expressed, but stable at low concentrations through the control of activation or inhibition by TIMP-2.

MMP-2 has a unique cell surface mode of activation in which it forms a ternary complex with MMP-14 (MT1-MMP) and TIMP-2. The N-terminal of TIMP-2, which has the inhibitory ability, binds to the catalytic domain of MMP-14, and the C'-terminal of TIMP-2 binds to the hemopexin-like domain of proMMP-2. Since MMP-14 anchors to the plasma membrane through the transmembrane domain and leaves the catalytic domain exposed extracellularly, the formation of this ternary complex allows proMMP-2 to localize at the cell surface. Once formed, it is cleaved and activated by a TIMP-free active MMP-14 molecule that localizes nearby [100]. Therefore, TIMP-2 is required for MMP-2 activation at low concentrations (Figure 1.3). As mentioned above, at high concentrations, TIMP-2 binds to a binding site of activated MMP-2 and reduces the activity of MMP-2. TIMP-2 also inhibits the activation of proMMP-2 by blocking all active MMP-14. Additional, several integrins, including alpha 2 beta 1 and alpha v beta 3, facilitate this process via anchoring the hemopexin domain of proMMP-2 on the cell surface.[184] It was reported that MMP-14 may activate alpha v beta 3 integrin through proteolytic cleavage.[45]

Following the initial removal of the pro-peptide to give a 64-kDa intermediate, the final processing to the fully active form of 62-kDa MMP-2 requires a further proteolytic cleavage at Asn80-Phe81 by another fully activated MMP-2 molecule.[193] Integrin alpha v beta 3 may promote this by providing a platform for autocatalytic interaction between the two MMP-2 molecules.[46] Mature MMP-2 can either bind to another cell surface anchor protein or be released. Besides the major activation pathway as described above, there are several other pathways that contribute to the activation of MMP-2. It was reported that proMMP-2 could be activated by physiological concentrations of uPA-plasmin through a mechanism independent of the action of other metallo-or acid proteinases.[139] Two coagulation factors, thrombin and factor Xa, have been

demonstrated to activate proMMP-2 in cultured VSMCs.[66,165] Rajagopalan et al. reported the activity of pro-MMP-2 is upregulated by ROS produced by macrophage-derived foam cells.[164] There is evidence that NF-κB, a critical inflammatory factor, induces the activation of MMP-2.[158,159] In addition, osteopontin deficiency led to a decreased activity of both MMP-2 and MMP-9,[20] and AngII-induced MMP-2 release from endothelial cells is mediated by TNF-alpha.[7] More recently, it was reported that in macrophages, oxidized LDLs (ox-LDLs) markedly elevated the levels of MMP-14 mRNA and protein.[166] However, the cross-talk between cell signaling pathways in MMP-2 activation is poorly understood.

The inhibition, catabolism, and clearance of MMP-2: The synthesis of MMP-2 is hardly suppressed at the transcription and post-transcription levels. Since MMP-2 is synthesized as a zymogen, the regulation of MMP-2 is mainly through regulation of the activity and/or activation of the enzyme. It should be kept in mind that proMMP-2 is stable under physiological conditions, i.e. the zymogen is not activated spontaneously. Mature MMP-2 molecules released from the cell surface may either maintain their active state or be inhibited by binding to TIMP-2, which is the endogenous inhibitor of MMP-2. The MMP-2/TIMP-2 complex retains 10% of proteolytic activity of MMP-2. In addition, at high levels, TIMP-2 regulates the activation of pro-MMP-2 by blocking all active MT1-MMP.[92] The fibronectin type II domain of MMP-2 interacts with thrombospondin properdin-like repeat (TSR) domain of thrombospondins (TSPs) to form a complex.[217] Endocytosis of the MMP-2/ TSP-2 complex, mediated by low density lipoprotein-related receptor protein (LRP) scavenger receptor, is another mechanism of MMP-2 control.[218] The LRP mediated endocytosis is regulated by a 39 kDa LRP-associated protein (RAP), which binds and antagonizes the function of LRP and other members of the LDL receptor family. LRP also mediates the clearance of the proMMP-2/TIMP-2 complex through a TSP independent pathway.[51] More recently, it was reported that furin, which is a serine protease, directly cleaves proMMP-2 within the trans-Golgi network (TGN)

to create an inactive form MMP-2 (63 kDa). This 63 kDa inactive MMP-2 cannot be further cleaved to an active form.[22]

Substrates of MMP-2

ECM components: The three-dimensional organization of the ECM molecules, including elastin, collagen, proteoglycans and structural glycoproteins, is optimal for the function of vessel walls. ECM components can be degraded and removed by proteolytic enzymes (especially MMPs) and cell (such as macrophages and fibroblasts) phagocytosis. MMP-2 has various ECM substrates including native collagen types (I, IV, V, VII, X, XI), denatured collagens (gelatin), elastin, fibronectin, laminin-5, aggrecan, brevican, neurocan, decorin, and vitronectin. Among these ECM molecules, fibronectin, vitronectin, and laminin are structural glycoproteins that have a multi-domain structure and enable interactions between cells and ECM constituents.[29] Collagen and elastin are the major protein components of vascular wall. The three repeats of fibronectin type II inserts in the catalytic domain of MMP-2 are required to bind and cleave collagen and elastin. [144]

Regulators of cell behavior: The ECM not only provides a structural framework to support cell adhesion and tissue integrity, but it also controls cell behavior by influencing signaling molecules. The ECM serves as a reservoir for many cytokines and chemokines that may affect cell adhesion and migration to their destined sites. When the ECM is degraded, these molecules are released. For example, TGF-beta can be released after degradation of the collagen-associated proteoglycan decorin, which is an anchor protein of TGF-beta.[95] Several MMPs (including MMP-2) may release TGF-beta through this pathway. MMP-2 and MMP-9 may proteolytically cleave and activate latent TGF-beta.[219] Moreover, MMP-2 may modulate the activity of fibroblast growth factor (FGF) by cleavage of the FGF receptor-1.[119] MMP-2, by binding to IGF and inhibiting its activity, may degrade insulin-like growth factor binding protein (IGFBP), which

may enhance the activity of IGF. In addition, MMP-2 is involved in the activation of endothelin-1 (ET-1). MMP-2 may cleave the Gly32-Leu33 bond of latent ET-1 (1-38) forming the potent vasoconstrictor ET-1 (1-32). This may increase cell adhesion ability through regulating endothelial expression of adhesion molecules such as E-selectin and intracellular adhesion molecule (ICAM).[58] Furthermore, active ET-1 produced by MMP-2 upregulates CD11b/CD18 expression on human neutrophils.[59] It was reported that MMP-2 was involved in the shedding of vascular cell adhesion molecule (VCAM-1) directly as well.[91]

Involvement in the expression and activation of other MMPs: It is known that the active form of MMP-2 can activate proMMP-9 on the surface of cancer cells. [65] On the contrary, Esparza et al. reported that the MT1-MMP/TIMP-2/MMP-2 complex, while facilitating activation of proMMP-2, could be involved in the regulation of MMP-9 expression.[53] Although these findings suggest MMP-2 may play several roles in the regulation of MMP-9 activity, the factors controlling these effects are largely unknown. MMP-2 also facilitates MMP-14 processing and activating the zymogen form of MMP-13.[111]

IV. Potential mechanisms of MMP-2 involvement in the development of atherosclerosis

Both secreted and active MMP-2 are elevated in human atherosclerotic lesions.[141,156] Due to lack of a selective MMP-2 inhibitor, MMP-2 deficient mice have been created to determine the role of MMP-2 in atherosclerosis. As mentioned above, MMP-2 deficient mice have reduced intimal hyperplasia in mouse carotid artery blood flow cessation model. [101,114]

MMP-2 is involved in the remodeling of the vascular wall

The ECM of the vascular wall is a complex network is comprised of many high molecular weight proteins and polysaccharides. These molecules are

organized according to which cells secreted them. The surrounding cells are also involved in the organization of ECM molecules. The three-dimensional structure of the ECM provides a support for cell adhesion, facilitating tissue integrity of vascular walls. Furthermore, the ECM may modulate signaling pathways that control numerous cellular behaviors including growth, differentiation, survival and morphogenesis.[128] The ECM also serves as a reservoir for growth factors and a binding site of lipoprotein, which contributes to the accumulation and oxidation of LDL particles in the arterial wall. Turnover of ECM plays a key role in cardiovascular diseases, including atherosclerosis and AAAs.[122] As mentioned previously, MMP-2 has various substrates of ECM components. Among these ECM molecules, collagen and elastin are the major protein components of the vascular wall. In particular, type I collagen is the major (60%) fibrillar collagen detectable in blood vessels.[137] Elastin is the most abundant protein in the large arteries, and strengthens the aortic wall against pulse waves. The disruption of elastin not only leads to dissection of arteries, but also contributes to proliferation of VSMCs and obstructive arterial diseases.[120] Other molecules involved are fibronectin, vitronectin and laminin, and structural glycoproteins which have a multi-domain structure and enable interactions between cells and ECM constituents.[29]

MMP-2 is involved in inflammatory cell adhesion and migration

Inflammatory cell recruitment and invasion are critical for the initiation and progression of atherosclerosis. Although macrophage accumulation is especially important for ox-LDL clearance, accumulation of lipid-laden foam cells results in increased size of atherosclerosis, cell death and necrotic core formation. MMP-2 facilitates cell migration in pathological processes.[69,114] In mice, MMP-2 deficiency has been shown to suppress VSMC migration from the media to the intima of the vessel wall.[101,114] Some studies suggest MMP-2 might influence neutrophil-endothelial adhesion by cleaving the latent endothelin-1 (ET-1),

creating the potent ET-1 and upregulating CD11b/CD18 expression.[58,59] Since the recruitment of macrophages is one of the initial steps of atherosclerosis development, MMP-2 might play an important role in the early stages of atherosclerosis.

MMP-2 is involved in platelet aggregation

Intravascular thrombosis is one of the most crucial pathological events in various cardiovascular diseases. Thrombosis deteriorates the hardening and narrowing of blood vessels as well as turbulent blood flow formation and therefore exacerbates vascular diseases. An injury to the surface of a blood vessel attracts platelet aggregation and leads to primary thrombus formation. Von Willebrand factor (vWF), a long-chain macromolecule, induces the activation of platelet glycoproteins (GPs) such as GPIb and GPIIb/IIIa (fibrinogen receptor). These glycoproteins mediate the mechano-chemical interactions between platelet-platelet and platelet-vascular wall and consequently lead to platelet adhesion and aggregation. During platelet aggregation, MMP-2 is translocated from the cytosol to the platelet surface and is released.[174] Solid phase vWF induces the release of MMP-2 from platelets. Human recombinant MMP-2 potentiated the effects of vWF on platelet adhesion via upregulating GPIb. An inhibition of MMP-2 reduced platelet adhesion when stimulated with svWF also suggests the important role of MMP-2 in platelet aggregation.[163] Thus, MMP-2 might be involved in atherosclerosis progression via influencing platelet aggregation and thrombus formation.

MMP-2 is involved in growth factor activity

The potential roles of growth factors in cardiovascular pathophysiology have raised considerable interest. For instance, it was reported that transplantation of bone marrow-derived cells containing T cells with dominant-

negative TGF-beta receptors led to a reduced size of atherosclerotic lesions but increased vulnerability to rupture in LDLr-/- mice.[73] MMP-2 is known to proteolytically cleave and activate TGF-beta.[95] MMP-2 might cleave decorin, which is an anchor protein of TGF-beta, thus releasing TGF-beta. [219] MMP-2 also increases the availability of insulin-like growth factor (IGF) by degrading the IGF binding proteins.[62] Furthermore, MMP-2 cleaves cell surface fibroblast growth factor (FGF) receptor-1 and releases the soluble FGF receptor into circulation. This soluble FGF receptor fragment is able to bind to FGF and might indirectly influence FGF availability. It is not known whether the removal of FGF receptors could influence cell response to FGF directly.

MMP-2 is involved in the activity of other MMPs

Other members of the MMP family are also involved in vascular diseases. At least 14 MMPs have been found in vascular cells. MMP-2 is involved in the activation of MMP-9,[65] which is another gelatinase in the MMP family and critical for atherosclerosis and AAA formation in some animal models. MMP-2 also facilitates MT-MMP to activate the zymogen form of MMP-13. There is evidence that MMP-13 affects the development of atherosclerosis as well.[127,99,161]

MMP-2 influences LDL modification and VSMC contraction

More recently, it was reported that MMP-2 was involved in the modification of LDL in arterial walls.[203] Furthermore, MMP-2 promotes aortic dilation by inhibiting the Ca^{2+} entry pathway of vascular smooth muscle contraction.[27]

V. Potential mechanisms of MMP-2 involvement in the development of AAAs

Both secretion and activation of MMP-2 are augmented in human

AAAs.[74,141] MMP-2 deficient mice have been used to determine the role of MMP-2 in AAAs. As mentioned above, abluminal application of $CaCl_2$-induced AAA formation was ablated in MMP-2 deficient mice.

MMP-2 is an elastiolytic enzyme

The hallmark pathology of AAAs is the progressive and irreversible degeneration of the medial elastin lamellae. This destruction of elastin may contribute to aneurysmal dilation and enlargement. Turnover of ECM plays a key role in AAAs.[8,122] As a major elastinase, MMP-2 is thought to be implicated in AAA formation.

MMP-2 is involved in inflammatory cell adhesion and migration

Inflammatory cell recruitment and invasion are critical for the initiation and progress of AAAs. A previous study by our lab, in hyperlipidemic mice, demonstrated that the accumulation of macrophages in the media of aortic wall after AngII-infusion is the earliest pathological event in AAA formation.[172] Similarly, a clinical trial study demonstrated that the concentration of MMP-2 was higher for small aneurysms as compared with large aneurysms, which suggests that MMP-2 might contribute to early aneurysmal dilation.[63] The potential role of MMP-2 in the early stages of AAAs might encourage the development of new approaches in the prevention of this disease.

Other potential roles MMP-2 might play in AAAs

MMP-2 may influence the apoptosis of vascular smooth muscle cells. The apoptosis of VSMCs is a prominent feature of AAAs. A modification in the activity of growth factors by MMP-2 might influence VSMC proliferation and apoptosis. Intravascular thrombosis is one of the major pathological events of AAA. As

described above, MMP-2 potentiates the vWF effects on platelet adhesion through up-regulating GPIb. [163] Therefore, MMP-2 might be involved in AAA progression via influencing platelet aggregation and thrombus formation.
Other members of MMP family are also involved in AAAs. As mentioned previously, MMP-2 is involved in the activation of MMP-9,[65] which is another member of the gelatinase sub-family and is critical for AAA formation in a couple of mouse models.

The primary hypothesis of this research is that MMP-2 plays an important role in the formation and progression of atherosclerosis and AAA in vitro and in vivo. This hypothesis was tested in four specific aims: (1) to determine whether MMP-2 deficiency is protective for atherosclerosis and angiotensin II-induced AAAs in apoE-/- mice; (2) to investigate the phenotypic change in aortas of MMP-2 deficient mice; (3) to investigate cell behavior of macrophages of MMP-2 deficient mice; (4) to determine whether bone marrow-derived MMP-2 is critical in atherosclerosis and AAA formation in vivo. The data will be presented over four chapters (Chapters 2-5) in this thesis.

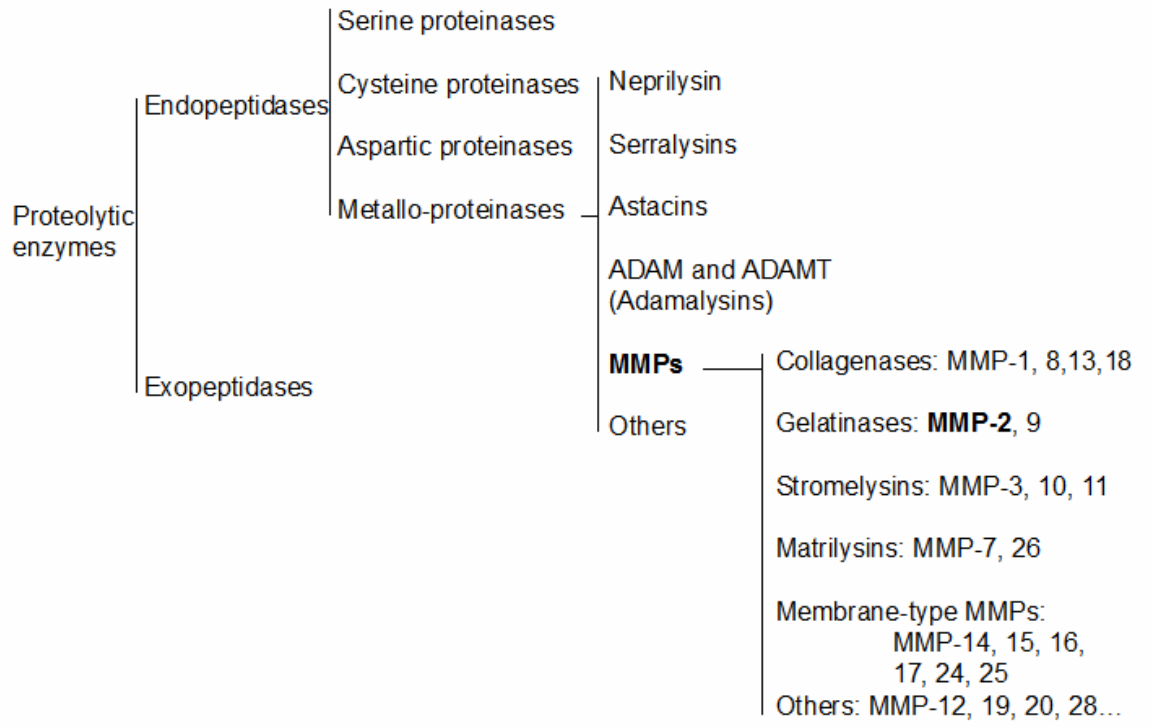


Figure 1.1 Classification of proteolytic enzymes

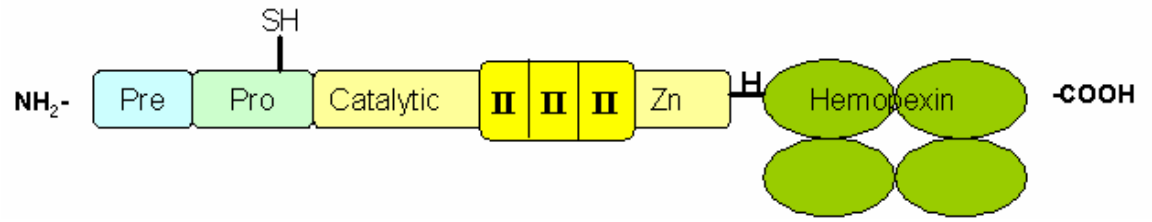


Figure 1.2 Domain composition of MMP-2. Pre - signal peptide which is removed after it directs synthesis to the endoplasmic reticulum; Pro – a pro-peptide with a zinc-binding SH group, which maintains enzyme latency until it is removed or disrupted; II - Fibronectin type II repeats, which are cysteine-rich and unique in MMP-2 and MMP-9; H - Hinge region; Zn - the catalytic domain contains the conserved zinc-binding region.

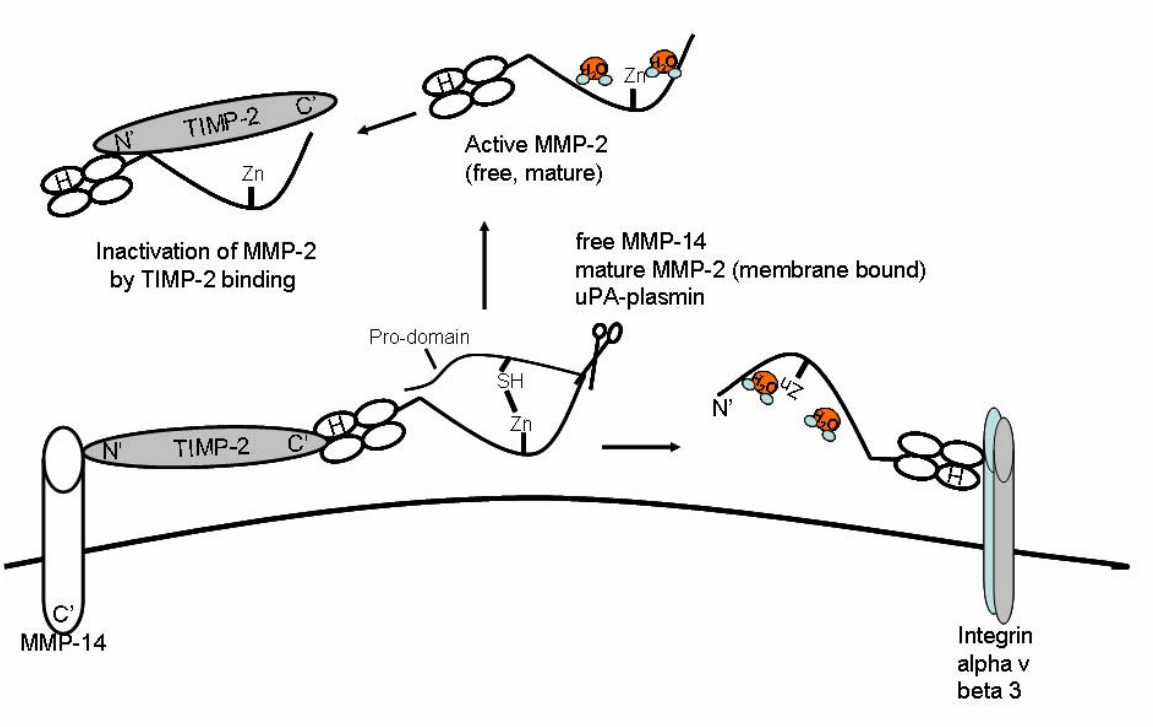


Figure 1.3 Activation of MMP-2 on the cell membrane MMP-2 has a unique cell surface mode of activation in which it forms a ternary complex with MT1-MMP (MMP-14) and TIMP-2. The N' terminal of TIMP-2 binds the catalytic domain of MT1-MMP, and the C'-terminal of TIMP-2 binds the hemopexin-like domain of pro-MMP-2. The formations of this ternary complex makes pro-MMP-2 localize at the cell surface and can be cleaved and activated by a TIMP-free active MT1-MMP molecule, which localizes nearby. Therefore, TIMP-2, well known as an endogenous inhibitor of MMP-2, is required for MMP-2 activation at low concentrations. At high concentrations, TIMP-2 binds to N'-terminal of activated MMP-2 and reduces the activity of the enzyme. Additionally, integrin αvβ3 is another molecule that may anchor proMMP-2 on the cell surface and favor MMP-2 maturation. It was reported that MT1-MMP might activate αvβ3 integrin through proteolytic cleavage.

Chapter Two

The Effects of MMP-2 Deficiency on Atherosclerosis and AngII-Induced AAAs in Apolipoprotein E Deficient Mice

I. Background

Apolipoprotein E deficient mouse model of atherosclerosis

Atherosclerosis, a progressive disease, might be the most important contributor to human mortality. Most studies of atherosclerosis in humans are performed after the disease is highly developed, when clinical signs or symptoms appear. In contrast, animal models permit investigators the opportunity to study the initiation and progression of preclinical stages of this disease. In order to provide essential information for treatment and prevention of the human disease, animal models should closely mimic the histopathologic changes in human disease as well as the interaction between pathogenetic factors and the disease process. In past experiments involving atherosclerosis, rabbits were the preferred model followed by non-human primates and swine. There are monetary disadvantages associated with the acquirement and maintenance of these animals, but more importantly, the scientific community is experiencing a moral shift away from the use of higher mammals as research models. The advent of the apoE deficient mouse model (referred to in this paper as apoE-/- or apoE knockout), created by a targeted inactivation of the apoE gene, provides a desirable research subject that is relatively cost effective for both experimental and breeding purposes.[160] Another hyperlipidemic model, LDL receptor deficient (LDLr-/-) mice, can have modest increased plasma total cholesterol levels, and require high fat diet to develop atherosclerosis. Because the pathology of atherosclerosis in these mice is not as well characterized as apoE-/- mice, LDLr-/- mice were not used in this dissertation project.

ApoE is a 34kD glycoprotein important for lipid metabolism. It is a component of all lipoprotein particles except low density lipoprotein (LDL). ApoE is a high affinity ligand of the LDL receptor, and the chylomicron-remnant receptor (LRP) on liver cells. Therefore, apoE is essential for the specific uptaking of apoE-containing particles by the liver.[221] ApoE also plays a key role in stimulating macrophage cholesterol efflux.[111] Although apoE -/- mice have normal body weights and maturation, they tend to develop hyperlipidemia and atherosclerosis spontaneously. To date, the apoE-/- mouse model is considered to be one of the most common models in the analysis of atherosclerosis. The atherosclerotic plaques of apoE-/- mice are extensive, reproducible and demonstrate a close similarity to human atherosclerotic lesions. For instance, lesions in both apoE-/- mice and humans have a tendency to progress from the fatty streak stage to the fibrous plaque stage with obvious fibrous caps and necrotic lipid cores. In addition to their histological similarities to humans, apoE-/- mice also exhibit an exacerbation in atherosclerosis response to environmental pathogenetic factors, such as smoking or a “western-type” diet, which contains 21% fat, 0.15% cholesterol, and no cholic acid.[145, 185] However, it should be kept in mind that apoE-/- mice have several features dissimilar to humans: lesions in the aortic root of apoE-/- mice are foam cell-rich, rather than smooth muscle cell-rich; plaque rupture is not observed in these mice whereas it occurs commonly and leads to heart attacks in humans; and most cholesterol is carried in VLDL, rather than in LDL as in humans.[98,130] Despite some differences, the atherosclerotic lesions formed in apoE-/- mice are remarkably similar to those found in human disease. Therefore, of all available animal models, the apoE deficient mouse has become widely used and accepted for research to identify the role of cytokines, chemoattractants and intercellular adhesion molecules in atherosclerosis.

Mouse model of AngII-induced AAAs

There are several mouse-models of chemical-induced AAAs. One such model uses the infusion of elastase into the infrarenal segments of mouse aortas.[162] A second model relies on periaortic administration of calcium chloride between the renal branches and the iliac bifurcation.[126] A more widely accepted model applies chronic infusion of AngII to induce reproducible AAA formation and accelerate atherosclerosis development in hyperlipidemic mice, including apoE-/- and LDLr-/- mice. The AngII infused mouse model mimics several similar features of AAAs in humans, such as a gender bias, luminal dilation, inflammatory cell recruitment, elastin fiber degeneration, and thrombi formation in AAA lesions. [126] There are two potential mechanisms by which AngII may induce AAAs. First, AngII-infusion might stimulate inflammatory cell infiltration via activating the NF-κB cascade and subsequently elevating certain cytokine levels, such as MCP-1 and VCAM-1. Second, AngII-infusion might enhance the activity of ECM degrading enzymes such as MMPs.[130] Also, a previous study demonstrated that the initial event in AngII-induced AAAs was the accumulation of macrophages in the media of the suprarenal aorta. This event has been associated with breaks in the elastin laminae, leading to thrombus formation and atherosclerosis in apoE-/- mice.[172]

The role of AngII in cardiovascular diseases

The renin-angiotensin-aldosterone system (RAAS) is one of the most critical in the regulation of hemodynamic homeostasis. When blood flow and pressure are reduced in the renal artery, renin is secreted by the juxtaglomerular apparatus (comprised of modified vascular smooth muscle of the afferent arteriole) and released into the blood. Renin is a protease which cleaves the inactive angiotensin I (AngI, 10 amino acid polypeptide) from a plasma protein (angiotensinogen). As AngI passes through the capillaries of the lungs and other organs, it is cleaved into AngII (an octapeptide) by angiotensin-converting

enzyme (ACE). Furthermore, AngII, which is the most biologically active component of the RAAS, is cleaved into fragments by aminopeptidases. These fragments of AngII include AngIII (2-8), AngIV (3-8) and some inactive fragments (4-8, 5-8, and so on).[131] In response to AngII and AngIII, the adrenal cortex synthesizes and releases aldosterone, which increases sodium and water retention by the collecting ducts, therefore, increasing blood volume and the blood pressure. In addition, AngII, directly and indirectly, enhances tubular reabsorption of sodium in proximal tubules as a consequence of glomerulotubular balance. AngII is a direct and potent vasoconstrictor of vascular smooth muscle by activating Ca^{2+} channels and releasing Ca^{2+} from the sarcoplasmic reticulum (SR). [151] Numerous clinical and laboratory data suggest that the RAAS is involved in the pathogenesis of atherosclerosis. The development of atherosclerosis in apoE-/- mice can be accelerated on a high fat diet or by infusion of AngII.[37] Multiple receptor subtypes exist for AngII including AT1a, AT1b, and AT2. It is generally assumed that AngII transduces most of its functions through AT1a receptors.[146,38]

AngII is not only implicated in hypertension, but also as a potent pro-inflammatory agent. In VSMCs, AngII is a potent stimulus for the generation of ROS.[77, 211] In addition, AngII upregulates the expression of many cytokines (including IL-1, IL-6, IL-8, MCP-1, TNF-alpha) and growth factors (including IGF, TGF-beta, PDGF, bFGF) that have been implicated in the pathogenesis of atherosclerosis. Furthermore, AngII induces production of adhesion molecules (including osteopontin, VCAM-1, ICAM-1 and E-selectin).[96,199] It was reported that AngII enhanced the expression of integrin alpha v beta 3, and that blocking antibodies to beta 3 attenuated AngII-induced adhesion in rat cardiac fibroblasts.[104] AngII induced leukocyte adhesion in both arterioles and venules mediated by P-selectin and some other cell adhesion molecules. AngII increases integrin beta 2 expression in peritoneal cells.[11] When elevated, MCP-1 (an important cytokine which is up-regulated by AngII) levels increased the CD11b positive monocyte/macrophage population in circulating blood of mouse.[197]

AngII plays multiple roles in the regulation of MMPs. It was reported that an antagonist of the AT1 receptor, irbesartan, reduced prostaglandin E2 (PGE2)-dependent MMP-2 activity, and this effect may in turn contribute to atherosclerotic plaque stabilization in humans.[31] In cultured rat peritoneal macrophages, an increased MMP-2 expression and activity can be elicited by AngII and this increase was prevented by losartan, which is an antagonist of the AT1 receptor.[121] AngII, through several pathways, might elicit an increase of MMP-2 activity indirectly. First, since the activity of pro-MMP-2 and pro-MMP-9 is upregulated by ROS produced by macrophage-derived foam cells,[164] AngII might induce the activation of these MMPs through upregulating ROS production. Second, it was reported that osteopontin deficiency led to a decreased activity of both MMP-2 and MMP-9.[20] This suggests that AngII may possibly increase MMP-2 activation via up-regulating osteopontin. Third, TGF-beta is a MMP-2 upregulator. When there is an AngII-induced increase of TGF-beta, the event may increase both expression and activation of MMP-2. Finally, it was reported that AngII-induced MMP-2 release from endothelial cells is mediated by TNF-alpha,[7] as well as that AngII increases TIMP-1 in rat VSMCs.[24]

Taken together, one of the major and unique advantages of using mice for experimental cardiovascular research is that it is possible to “knock out” or replace endogenous genes, thereby determining the influence of protein expression on the development of diseases. In this study, our hypothesis is that MMP-2 deficiency might reduce atherosclerosis and AngII-induced AAA development in an apoE deficient mouse model.

II. Materials and Methods

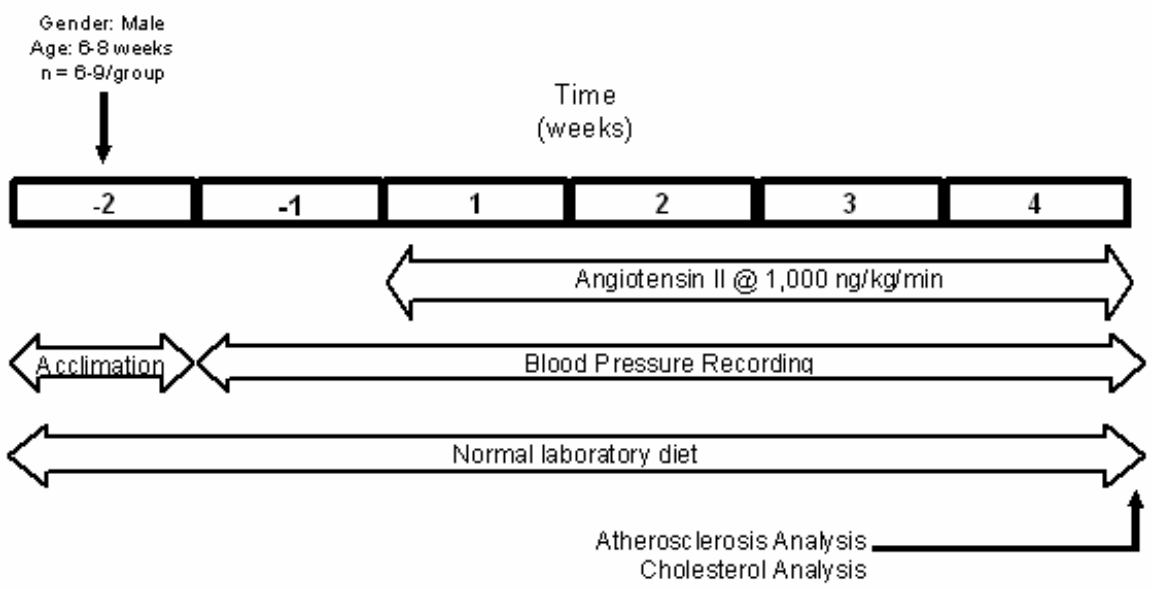
Animals

MMP-2 deficient mice (C57BL/6 background) were obtained from Dr. Itohara (Brain Science Institute, RIKEN, Saitam, Japan). ApoE-/- mice on a C57BL/6 background were obtained from the Jackson Laboratory (Bar Harbor,

ME, USA). All of the mice were housed under barrier conditions with normal laboratory diet and water ad libitum. MMP-2-/- mice and apoE-/- mice were then bred and their offspring, generation F1 (MMP-2+/- x apoE+/-), were mated to produce littermates with the desired MMP-2+/+ and MMP-2-/- x apoE-/- mice genotypes (F2). Male MMP-2+/+, +/- and -/- x apoE-/- littermates were used in all subsequent experiments. All procedures were performed with the approval of the University of Kentucky Institutional Animal Care and Use Committee.

Experimental protocol

In order to address the issues raised by this specific aim, my experimental protocol will be, primarily, two-fold. First, to investigate AngII-induced AAAs and atherosclerosis in MMP-2+/+, +/-, and -/- x apoE-/-, I designed the experiment as follows:



Second, I will use 20 to 24-week-old male apoE-/- mice with various MMP-2 genotypes to investigate atherosclerosis development in aged hyperlipidemic animals. These mice will be fed with normal laboratory diet and will not receive AngII infusion.

Polymerase chain reaction (PCR) for mouse genotyping

Genotyping for MMP-2 deficiency: PCR was performed using genomic DNA isolated from tail snip samples of mice using a DNeasy tissue kit (Qiagen Inc., Valencia, CA, USA). The exon1 of the MMP-2 gene was exchanged to reverse pgk-neo gene in MMP-2-/- mice. The amplification was performed using these specific primers: for the mutant allele, the forward primer recognized the DNA sequencing on the reverse pgk-neo gene (5'-TGCAAAGCGCATGCTCCAGA-3'). The reverse primer recognized the DNA sequence on exon2 (5'-TGTATGTGATCTGGTTCTTG-3'). The amplicon is 1.1Kb (kilo-base pair) fragment. For the wild type allele, both primers recognize DNA sequences on the exon1 of MMP-2. The forward primer reads 5'-CAACGATGGAGGCACGAGTG-3' and the reverse 5'-GCCGGGGAACTTGATGATGG-3'. The amplicon is a 120bp (base pair) fragment. The information for these primers was kindly provided by Dr. Itoh (Institute for Virus Research, Kyoto, Japan). The thermocycling reactions were performed as follows: 35 cycles of denaturation at 94°C for 1 minute, annealing at 57°C for 1 minute, extension at 72°C for 1 minute, followed by 1 cycle of final elongation at 72°C for 7 minutes.

Genotyping for ApoE deficiency: To detect the apoE deficiency, PCR was performed as described previously.[160] The sequences of each primer were: 5'-GCCGCCCCGACTGCATCT-3'; 5'-TGTGACTTGGGAGCTCTGCAGC-3'; and 5'-GCCTAGCCGAGGGAGAGCCG-3'. Amplification of a single species of PCR product of 245 bp (mutant allele) and/or 155 bp (wild type allele) corresponded to a genotype. The thermocycling reactions were performed as follows: the initial denaturation at 94°C for 1 minute, followed by 35 cycles of denaturation at 94°C for 1 minute, annealing at 55°C for 1 minute, extension at 72°C for 1 minute, then followed by 1 cycle of final elongation at 72 °C for 5 minutes.

Gelatin zymography

Gelatin zymography was performed to confirm the disruption of the MMP-2 gene as described previously.[83] Frozen segments of mouse aorta were homogenized in Rho-binding lysis buffer (RLB) containing Tris 50mM (pH7.2), NaCl (150mM), $MgCl_2$ (10mM), 1% Triton X-100, 0.1% SDS and 0.5% Sodium deoxycholate for 30 minutes on ice. Following extraction, the homogenate was centrifuged at 13,000 x g for 10 minutes at 4 °C. The protein content was measured by a DC-protein assay kit (BioRad Lab., Hercules, CA, USA). A 7.5% SDS-polyacrylamide gel containing 2% gelatin was pre-run at 50 volts for 5 to10 minutes. Protein extracts of aortas or lysis of mouse peritoneal macrophages (MPMs) (15µg) and an appropriate amount of 5x non-reduced loading dye were loaded onto the gel. The gel was run at 50 volts until samples were through stacking gel, then the voltage was increased to 120 volts. After the electrophoresis, the gel was soaked in a 2.5% Triton X-100 solution for 1 hour at room temperature (RT) on a shaker to remove SDS. Then the gel was washed twice with distilled H2O (15 minutes per wash) at RT on a shaker. After washing, the gel was incubated overnight at 37°C in 50 mM Tris buffer containing either 5 mM $CaCl_2$ or 10 mM EDTA (negative control for MMP activity) on a shaker. Then gel was stained with Brilliant Blue R (Sigma, St. Louis, MO, USA) for 15 to 30 minutes at RT. The gel was de-stained with 1x de-staining solution containing 1% glycerol, 7.5% acetic acid, 30% methanol, and 61.5% H_2O, until clear bands appear. Bands of gelatin lysis by MMP-2 were quantified using a Kodak 440CF image station.

AngII infusions and blood pressure measurement

For AngII infusion, mice were anesthetized with sterile saline containing 22.7% ketamine and 3.4% xylazine. AngII (1,000 ng/kg/min) or saline was administered subcutaneously via Alzet osmotic minipumps (DURECT Corp., Cupertino, CA, USA) as described previously.[37]

Systolic blood pressures were obtained from conscious mice using a computerized tail cuff method (BP-2000 Visitech Systems, Apex, NC,USA), as previously described.[38] Mice were acclimated to the system for two weeks prior to the minipump implantation, and measurements were obtained at the same time of day throughout the study, 5 days per week.

Peripheral white blood cell counting

Blood was obtained by retro-orbital bleeding of anaesthetized mice using micro-hematocrit capillary tubes (Fisher scientific, Pittsburgh, PA, USA) and was blown into microtainer tube with EDTA (Becton Dickinson & Co., Franklin Lakes, NJ, USA). Peripheral blood cells were measured using a Coulter counter (Beckman Coulter Inc. Miami, FL, USA).

Serum lipids and lipoprotein determination

Mouse serum total cholesterol concentrations were determined with cholesterol enzymatic assay kits (Wako Chemical, Richmond, VA, USA). The results were read on a 96-well plate with a microplate reader (Bio-Rad Model 550) at 490nm.

Lipoprotein cholesterol distributions were evaluated in individual serum samples (50 µl) from 5 to 7 mice in each group after fractionation by size exclusion chromatography on a single Superose 6 column.[38] Fractions were collected and cholesterol concentrations were determined with an enzymatic based kit (Wako Chemicals USA Inc., VA, USA). The 96-well plates were read on the microplate reader at 490nm.

Quantification of atherosclerosis

Quantification of lesions in aortic root sections*:* Atherosclerotic lesions were quantified in the aortic root as described previously.[40] In brief, the upper

third portion of the heart was embedded and frozen in OCT (optimal cutting temperature) compound (Sakura Finetec USA Inc., Torrance, CA, USA). The heart was then sectioned on a cryostat from the direction of the apex toward the aortic valves until the aortic valves or cusps were observed within the aortic sinus. Beginning at this point, sections were cut at a thickness of 10 μm and collected sequentially on eight slides, 9 sections on each slide. A total distance of approximately 800 μm was taken for analysis. Sections were stained for neutral lipids using Oil Red O (Sigma), and counter stained with hematoxylin (Biomeda corp., Foster City, CA, USA). Digital images were taken and lesions were analyzed using ImagePro software version 4.1 (Media Cybernetics, Silver Spring, MD, USA).

En Face quantification of atherosclerosis: The percentage of the intimal area covered by atherosclerotic lesion was quantified in the aortic arch as described previously.[40] Aortas were fixed in 4% paraformaldehyde overnight. After the periadventitial fat and connective tissue around aortas was removed, aortas were cut and pinned on black paper placed over wax plates to expose the intimal surface. Digital images were captured and atherosclerosis was measured as a percentage of lesion area to total surface area using ImagePro software.

Classification of AAAs

AngII-induced AAAs were classified into four classes based on a standard which is described previously.[38] Type I AAA is defined as having a dilated lumen in the supra-renal region of the aorta without thrombus. A Type II AAA is defined as remodeled tissue in the supra-renal region that contains thrombus. Type III AAA is defined as multiple aneurysms containing thrombus. The most severe occurrence is a Type IV AAA and is defined as rupture that leads to death of mouse (Figure 2.5).

Immunohistochemistry staining

Air-dried cryostat (8μm thick) sections were fixed in cold acetone for 10 minutes, then were incubated with 1% hydrogen peroxide (H_2O_2) for 2-5 minutes at 37°C, then were incubated in 5% blocking serum for 15-30 minutes, and with respective antibodies: chicken anti-mouse MMP-2 (50μg/ml), MMP-14 (2.5μg/ml), and TIMP-2 (10μg/ml) antibodies (Aves Labs, Inc., Tigard, OR, USA). Respective pre-immune antibodies were used as negative controls. After washing, the sections were incubated in biotinylated rabbit anti-chicken secondary antibody (1:1,000; Jackson ImmunoResearch Lab Inc., West Grove, PA, USA).

For macrophage staining, a rabbit anti-mouse macrophage antibody (1:1,000; Accurate Chemical & Scientific Corp., Westbury, NY, USA) was incubated for 15 minutes at 40°C, then incubated in biotinylated goat anti-rabbit secondary antibodies (1:500; Vector Lab Inc., Burlingame, CA, USA). After washing, the sections were incubated in Elite Vectastain ABC solution (Vector Lab. Inc.) for 10 minutes at 40°C, then washed with peroxidase enhancer buffer (Biomed Corp.), then incubated with peroxidase chromogen (AEC; Biomed Corp.). After washing, the slides were counter stained with hematoxylin and mounted with glycerol gelatin (Sigma diagnostic Inc.).

Collagen staining using Gomori Trichrome

Sections (8 μm) of frozen tissue (aortic root) were fixed in cold acetone for 10 minutes and washed with distilled water. The slides were incubated with bouin solution (a mixture of saturated picric acid (75 ml), 37-40% formaldehyde (25 ml), and glacial acetic acid (5 ml)) for 1 hour at 56°C. After washing with dH_2O until yellow color disappears, the slides were incubated with working solution of Weigert hematoxylin containing equal amounts of solution A (a mixture of hematoxylin (0.5ml) and 95% alcohol (50 ml)) and solution B (a mixture of concentrated hydrochloric acid (5 ml), 29% ferric chloride solution (20 ml), and distilled H_2O (47.5 ml)) for 10 min at RT. After rinsing with dH_2O, the slides were

incubated with Gimori solution for 15 to 20 minutes, then differentiated in 0.5% acetic acid for 2 minutes at room temperature. After rinsing with ethanol, the slides were rinsed again with water and mounted with permanent mounting medium (H5000, Vector lab. Inc.).

Statistics

One-way ANOVA was performed to test the equality of three or more means with one independent variable at one time by using variances. A Student's t-test was used to test the difference between two groups. All of the statistical analyses were done with SigmaStat 2.03 software (SPSS Inc., Chicago, IL, USA). Values with $P < 0.05$ were considered statistically significant.

III. Results

Confirmation of the genotype of MMP-2 of mice

PCR was performed to examine the MMP-2 genotype of mice. The 1.1 kb amplified DNA product of mutant allele was detected only in DNA from MMP-2-/- mice and not in the DNA from MMP-2 wild type control mice (Figure 2.1 A). Gelatin zymography of aortic extracts was performed to confirm MMP-2 genotypes of mice. As predicted, the band of gelatin lysis by MMP-2 was not visualized in the extracts of aortas of MMP-2 deficient mice (Figure 2.1 B).

Effects of MMP-2 deficiency on AngII-induced AAAs and atherosclerosis in hyperlipidemic mice

Effect on body and organ weight: MMP-2 -/- x apoE-/- mice showed a tendency to have lower body weight when compared with MMP-2 +/+ x apoE-/- mice before AngII infusion. However, there was not a statistically significant difference ($P = 0.082$). After AngII-infusion for 28 days, a significantly lower body

weight was observed in MMP-2 deficient mice as compared to MMP-2 wild type mice ($P < 0.05$; Table 2.1). No significant difference was detected between the body weight of MMP-2 +/- vs. either MMP-2 +/+ or -/- mice. There was a correspondingly lower weight of the spleens from MMP-2-/- mice as compared with spleens from MMP-2 +/- mice and MMP-2 +/+ mice at the end of the experiment ($P < 0.013$; Table 2.1). However, the ratio of spleen to body weight of these mice was similar. The livers of MMP-2-/- mice exhibited this trend as well, however statistical significance was not achieved.

Effect on blood pressure: Mini-pumps filled with AngII were implanted subcutaneously into mice. There was a significant increase of blood pressure caused by AngII infusion in all of the three groups of mice with various genotypes of MMP-2. However, no significant difference was detected among various MMP-2 genotypes at both basal level and after AngII-infusion for 28 days (Table 2.1). The increase of blood pressure level induced by AngII-infusion was similar in each MMP-2 genotype mice group (MMP-2+/+ 33.5 ± 11.3 vs. +/- 43.0 ± 8.7 vs. -/- 40.7 ± 2.6 mmHg).

The effect on serum cholesterol levels: Serum total cholesterol concentrations were measured. There was a tendency of lower total cholesterol (TC) level in MMP-2-/- mice compared with MMP-2 +/- and +/+ x apoE-/- mice ($P = 0.064$; Table 2.1). Lipoprotein distribution was measured using size exclusion chromatography. The curves of VLDL, LDL/IDL and HDL of mice with various MMP-2 genotypes were overlapped with no significant difference (Figure 2.2).

The effect on atherosclerotic lesion area: The extent of atherosclerosis was quantified with both en face analysis of the aortic arch and sequential cross sectioning of the aortic root as described previously. Surprisingly, the area of atherosclerotic lesion was similar among mice with various MMP-2 genotypes. The percent atherosclerosis area on the intimal surface of aortic arch was 2.2 ± 0.5% vs. 3.9 ± 1.6% vs. 3.1 ± 0.8% in MMP-2-/-, +/- and +/+ x apoE-/- mice, respectively (Figure 2.3). On the transverse sections of aortic root, the size of atherosclerotic plaque was 12.9 ± 5.7 x 10^{-3} mm^2 vs. 3.9 ± 1.1 x 10^{-3} mm^2 vs.

10.1 ± 3.8 x 10^{-3} mm^2 in MMP-2-/-, +/- and +/+ x apoE-/- mice, respectively (Figure 2.4).

The effect on AngII-induced aneurysms: AngII infusion (1,000 ng/kg/min) for 28 days generated AAAs in 50% of MMP-2 +/+ mice (3 out of 6), 67% of MMP-2 +/- mice (6 out of 9) and 67% of MMP-2 -/- mice (4 out of 6), respectively (Figure 2.6). There was no statistical difference among the three groups. Two of MMP-2+/- mice died of AAA rupture at the 6th and 23rd day of AngII infusion, respectively. One MMP-2-/- mouse died of AAA rupture at the nineteenth day of AngII infusion. No AAA rupture occurred in MMP-2+/+ mice. Abdominal aortas were weighed to provide an index of AAA severity. The weight of abdominal aortas was similar in each MMP-2 genotype mice group (19.7 ± 6.1 mg vs. 16.9 ± 3.3 mg vs. 18.2 ± 3.0 mg in MMP-2+/+, +/-, and -/- x apoE-/- mice, respectively; Figure 2.7).

Effects of MMP-2 deficiency on atherosclerosis in 5 month-old ApoE-/- mice

General parameters: There was not a significant difference in the body, liver or spleen weight among MMP-2+/+, MMP-2+/-, and MMP-2-/- x apoE-/- mice. Interestingly, the number of peripheral white blood cell (WBC) was dramatically raised in MMP-2-/- x apoE-/- mice as compared to MMP-2+/+ and +/- x apoE-/- mice (Table 2.2).

The effect on atherosclerotic lesion area: The deficiency of MMP-2-/- did not decrease the size of atherosclerosis in AngII-infused apoE-/- mice: No protective effect from MMP-2 deficiency was detected concerning the size of atherosclerotic lesions. Actually, the size of atherosclerotic plaque tended to increase in MMP-2-/- apoE-/- mice (54.1 ± 20.5 x 10^{-3} mm^2 vs. 11.0 ± 4.4 x10^{-3} mm^2 vs. 18.5 ± 15.3 x 10^{-3} mm^2 of the lesion area on the transverse sections of aortic root in MMP-2-/-, +/-, and +/+, respectively; Figure 2.8). However, the difference was not statistically significant. Interestingly, the deletion of the MMP-2 gene dramatically changed the cellular and ECM components of the atherosclerotic plaques. The histopathologic and immunohistochemical staining

demonstrated that collagen was increased as well as cellular content. Both macrophages and smooth muscle cells were reduced in plaques of MMP-2-/- mice as compared to plaques of MMP-2 wild type mice (Figure 2.9). Immunostaining also indicated a relative reduction of MMP-14 and TIMP-2 expression in atherosclerotic lesion of MMP-2-/- x apoE-/- mice.

IV. Discussion

Twelve-week-old MMP-2-/- x apoE-/- mice showed lower body weight as compared with MMP-2+/+ x apoE-/- mice. However, this difference in body weight was not detected in 5-month-old apoE-/- mice. It was reported that the inhibition of MMP-2 impairs adipose tissue growth via the formation of a collagen-rich matrix cap in mice.[123] On the basis of our data, we concluded that MMP-2 deficiency might retard but not ablate the development of adipose tissue of apoE-/- mice. In future study, the weight of adipose tissue should be measured. MMP-2 deficiency did not influence AngII-induced hypertension in apoE-/- mice.

MMP-2 has been shown to degrade ECM components of the vascular wall and become highly activated in AAAs and atherosclerotic lesions. It was reported that MMP-2 deficient mice on a C57BL/6 background were resistant to CaCl2-induced AAAs formation. [126] Thus, it was surprising that MMP-2 deficiency did not reduce the size of atherosclerotic lesions or the incidence of AngII-induced AAAs in apoE deficient mice. There are a few mechanisms that may account for this discrepancy. First, MMP-2 might be insignificant in the progression of AAAs and atherosclerosis and second, the increment of other pathogenic factors might overwhelm the beneficial effect of MMP-2 deficiency in apoE-/- mice. Several hypotheses might account for the latter mechanism.

MMP-2 deficiency might cause a compensatory over-expression of one or more other elastinases in MMP-2-/- mice. As mentioned previously, MMP-9, another member of gelatinase family, has been implicated in atherosclerosis and AAAs. An adaptive mechanism might be activated in MMP-2 deficient mice, thus increasing MMP-9 expression.

Another reason may be that AngII infusion might enhance the production of other elastinases. AngII is not only implicated in hypertension, but is also a potent pro-inflammatory factor that has significant atherogenic actions via multiple pathways. There are several mechanisms by which AngII may increase the activity of MMP-9 in the vessel wall. AngII is a potent stimulus for the generation of ROS,[77,211] which upregulates MMP-9 activation in the vascular wall.[164] Also, AngII induces activation of MMP-9 via upregulating osteopontin,[199] the deletion of which can cause a decrease in MMP-9 activity in apoE-/- mice.[20] Finally, AngII induces production of many inflammatory cytokines. Some of these cytokines, such as MCP-1, IL-1, TNF-alpha, may upregulate MMP-9 activity.[30]

AngII upregulates the expression of many growth factors including IGF, TGF-beta, PDGF, and bFGF. These growth factors have been implicated in the pathogenesis of atherosclerosis. Furthermore, AngII induces production of adhesion molecules (including osteopontin, VCAM-1, ICAM-1 and E-selectin, integrin alpha v beta 3, integrin beta 2).[11,104,199] Elevated MCP-1, an important cytokine that is upregulated by AngII, increases the CD11b positive monocyte/macrophage population in mouse models.[197]

Hyperlipidemia might increase the activity of MMPs other than MMP-2. There is plenty of evidence suggesting that lipid lowering by either low-cholesterol diet or lipid-lowering drugs leads to reduced activity of MMPs, including MMP-9, reducing the extent of aortic dilation and increasing the stability of atherosclerotic plaques in animal models. [2,189] Furthermore, the hormone/cytokine-like anti-atherogenic effects of apoE on the surrounding cells of the vascular wall should be considered in the current experiment in which apoE null mice were used. It was realized that the anti-atherogenic roles of apoE are beyond the regulation of lipoprotein metabolism and transport. Although the mechanism is not completely understood, it was reported that apoE can suppress the activation and proliferation of T lymphocytes via inhibiting important signaling such as intracellular calcium accumulation and phosphatidylinositol (PDI) turnover. [90] As a consequence, apoE inhibits the production of interleukin (IL)-2.[106] ApoE also inhibits SMC proliferation and migration via binding to

LRP.[223] Therefore, besides leading to hyperlipidemia in the mouse model, the deficiency of apoE might exacerbate vascular diseases directly.

In summary, apoE deficiency and exogenous AngII might have synergetic or additive pro-atherogenic effects on the atherosclerosis, overwhelming any benefits the deletion of MMP-2 might create. Based on data from our non-AngII treated MMP-2-/- x apoE-/- mice, we concluded that apoE deficiency might negate any protective effects MMP-2 deficiency might provide in the initiation and maturation of atherogenesis. Interestingly, the cellular and ECM content of the atherosclerotic plaque were changed in MMP-2 deficient apoE-/- mice. This suggested that the cell infiltration and ECM degradation during the pathologic process of atherosclerosis were influenced by MMP-2 in spite of the possible upregulation of the cytokines, growth factors, and other proteinases in the vascular wall. The less pronounced immunohistochemical staining of TIMP-2 and MMP-14 might be due to the reduced cellular content in the plaques in MMP-2-/- mice. Further study is required to quantify these changes and evaluate the significance of the difference.

The current findings of this study make it essential to investigate the phenotypic changes in the vascular wall and inflammatory cells of MMP-2 deficient mice. The hypothesis, concerning increased expression of other MMPs, namely MMP-9, and the subsequent morphological changes in vascular wall and associated inflammatory cells in the absence of MMP-2, will be presented and evaluated in the next two chapters.

Table 2.1 General parameters of AngII-infused 12-week-old male apoE-/- mice with various MMP-2 genotypes

Mouse	MMP-2+/+ x apoE-/-	MMP-2+/- x apoE-/-	MMP-2-/- x apoE-/-
Body weight before AngII infusion (g)	24.2 ± 0.9	24.4 ± 0.5	21.1 ± 1.7
Body weight at the 28th day of AngII infusion (g)	26.1 ± 0.6	25.7 ± 0.5	23.3 ± 1.1*
Body weight gain during AngII infusion (g)	2.0 ± 0.6	1.3 ± 0.4	2.2 ± 0.8
Spleen weight (x10^{-2} g)	9.8 ± 0.3	9.9 ± 0.6	7.7 ± 0.5*
Liver weight (g)	1.26 ± 0.05	1.15 ± 0.07	1.03 ± 0.08
Spleen/body weight (%)	0.38 ± 0.02	0.38 ± 0.02	0.33 ± 0.01
Liver/body weight (%)	4.8 ± 0.1	4.5 ± 0.3	4.4 ± 0.3
Total cholesterol (mg/dl)	282 ± 12	230 ± 33	221 ± 12
Blood pressure before AngII infusion (mmHg)	137 ± 10	124 ± 6	120 ± 4
Blood pressure at the 28th day of AngII infusion (mmHg)	159 ± 9†	167 ± 6†	161 ± 5†

Data represent mean ± SEM for groups of 6-9 mice.
*P<0.05 with respect to MMP-2+/+ x apoE-/- mice
† P<0.05 with respect to mice before AngII infusion

Table 2.2 General parameters of 5-month-old male apoE-/- mice with various MMP-2 genotypes

Mouse	MMP-2+/+ x apoE-/-	MMP-2+/- x apoE-/-	MMP-2-/- x apoE-/-
Body weight (g)	29.0 ± 1.0	30.6 ± 1.0	28.6 ± 0.7
Spleen weight ($x10^{-2}$ g)	10.2 ± 0.3	10.9 ± 1.5	14.8 ± 2.8
Liver weight (g)	1.24 ± 0.08	1.24 ± 0.07	1.28 ± 0.06
WBC ($1x10^3$ cells/mm^3)	7.1 ± 1.6	6.3 ± 0.6	13.1 ± 1.2*

Data represent mean ± SEM for groups of 3-6 mice.
*P<0.05 with respect to MMP-2+/+ x apoE-/- mice.

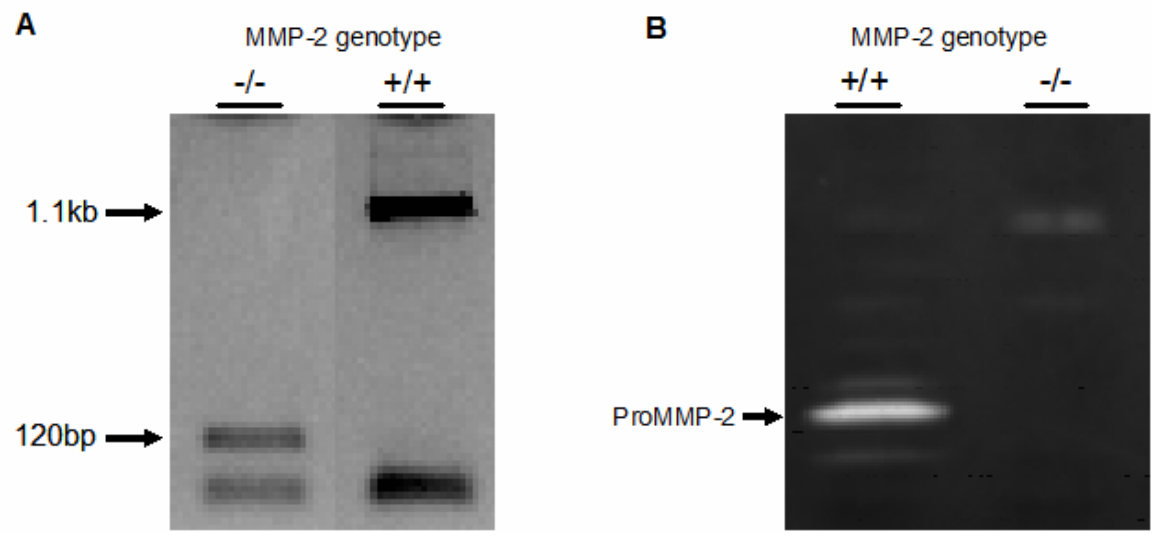


Figure 2.1 Confirmation of MMP-2 genotype of mice (A) Representive PCR for MMP-2 genotyping is shown. The 1.1 kb band (for mutant allele) was detected in DNA of MMP-2-/- mice and was completely absent in DNA of the wild type control mice. The 120 bp band (for wild type MMP-2 allele) was detected in DNA of wild type mice but not in DNA of MMP-2 -/- mice. (B) Gelatin zymography of extracts (15µg) of aortas of MMP-2+/+ and -/- x apoE-/- mice. No MMP-2 expression was detected in MMP-2 deficient mice, whereas a prominent 72kDa band of gelatin lysis by MMP-2 was present in the MMP-2 wild type mice.

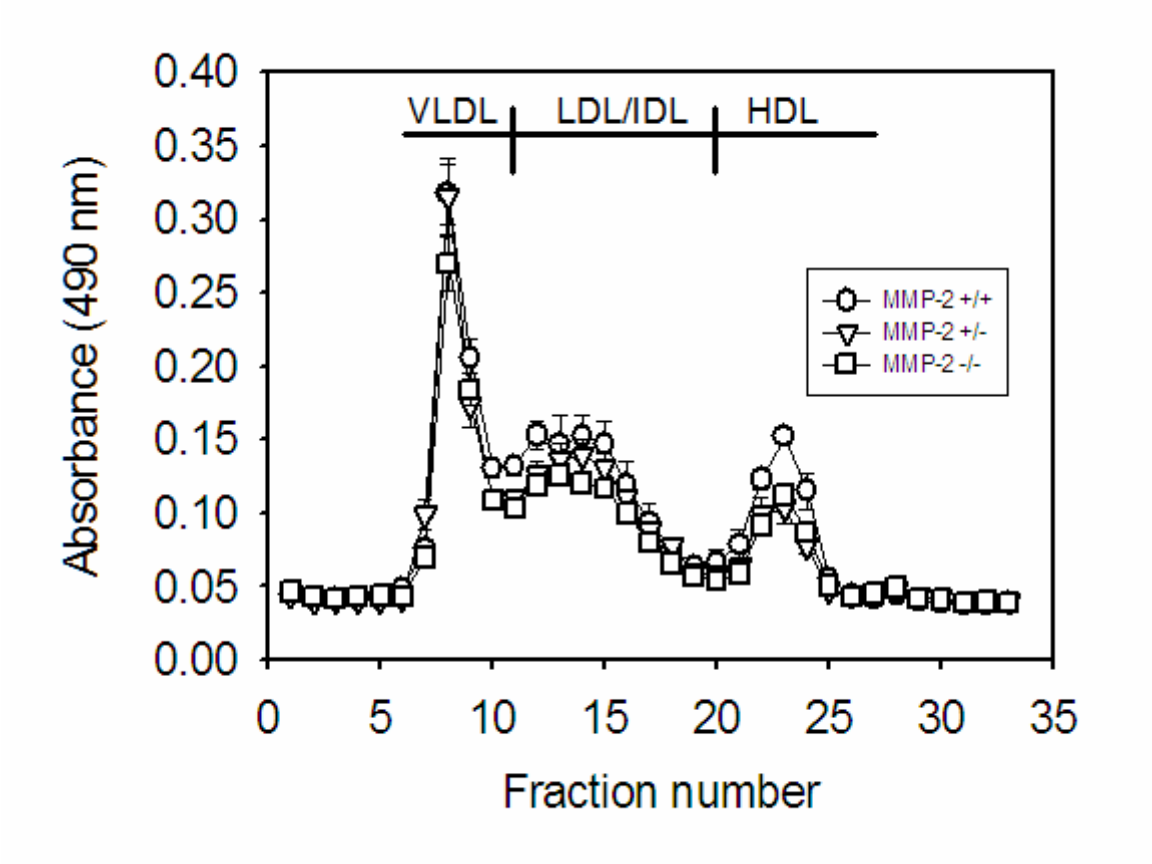


Figure 2.2 Effect of MMP-2 genotype and AngII infusion on the lipoprotein distribution of 12-week-old apoE-/- mice Lipoprotein distribution of mice was resolved using size exclusion chromatography, namely, fast performed lipid chromatography (FPLC). The curves of VLDL, LDL/IDL and HDL of the mice were overlapped. Points represent the means of 5-7 individual mice, and bars are SEM.

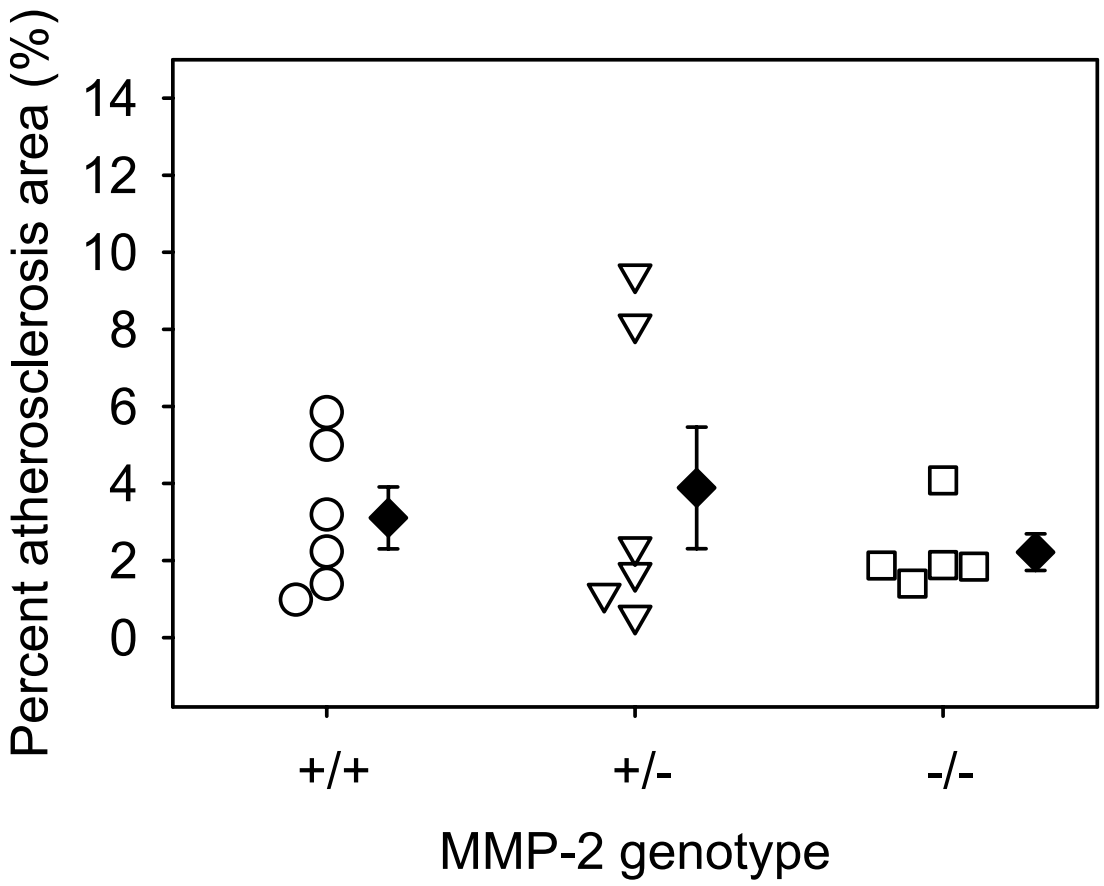


Figure 2.3 Effect of MMP-2 genotype on AngII-induced atherosclerosis on aortic arch of 12-week-old apoE-/- mice The percentage of the intimal area covered by atherosclerotic lesion was quantified in the aortic arch. The aortas from mice that died from AAA rupture were not included. There was not a significant difference in atherosclerosis among apoE-/- mice with various MMP-2 genotypes. Values obtained from individual mice are represented in open symbols, the means are depicted as closed symbols and SEM as bars.

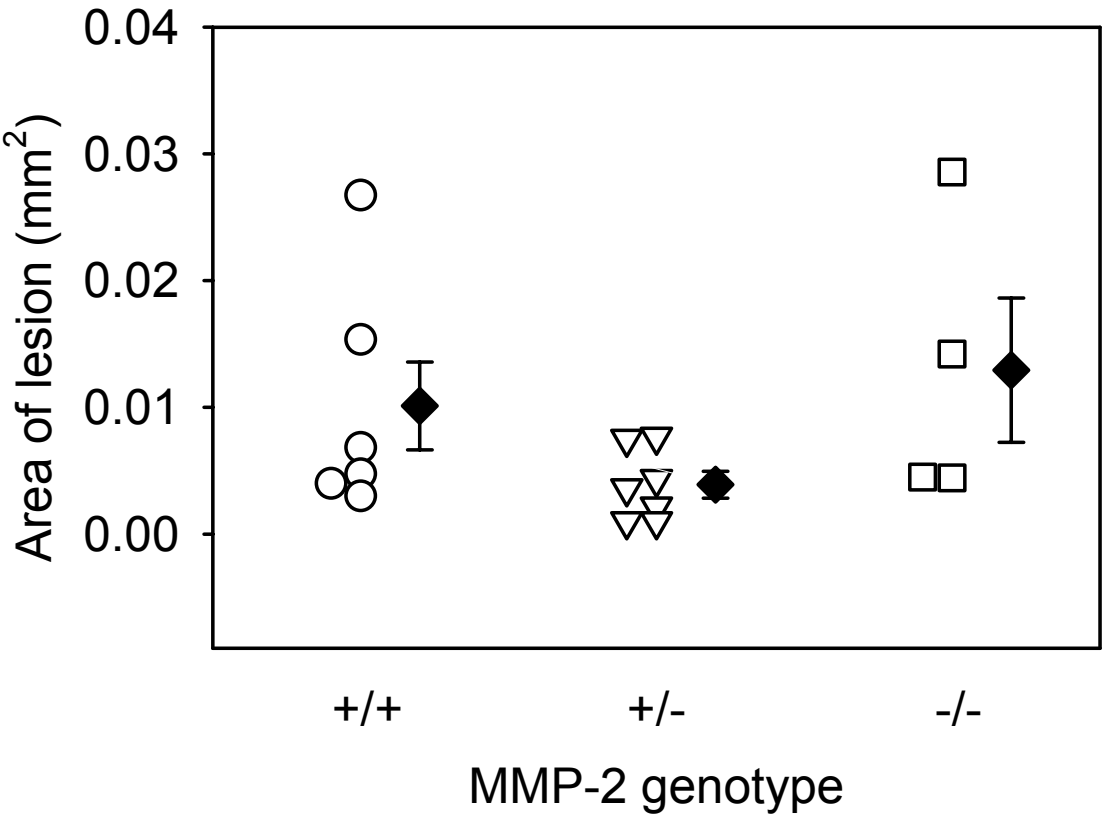


Figure 2.4 Effect of MMP-2 genotype on AngII-induced atherosclerosis on aortic root of 12-week-old apoE-/- mice Area of atherosclerosis was measured in the sections of aortic root of mice. Lesion area was determined in 10 μm-thick sections at 80 μm intervals after Oil Red O staining. Values obtained from individual mice are represented in open symbols, the means are depicted as closed symbols and SEM as bars.

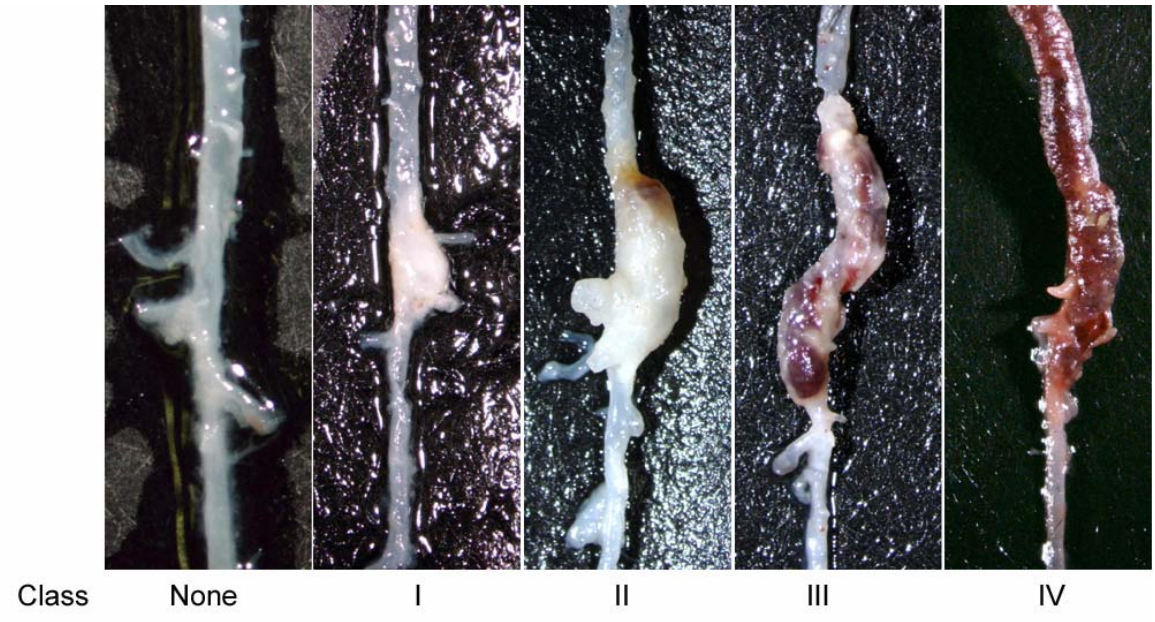


Figure 2.5 Classification of AngII-induced AAAs. Type I AAAs is defined as dilated lumen in the supra-renal region of the aorta without thrombus. Type II AAAs is defined as remodeled tissue in the supra-renal region that contains thrombus. Type III AAAs is defined as multiple aneurysms containing thrombus. Type IV AAAs is defined as rupture of AAAs which led to death of mouse.

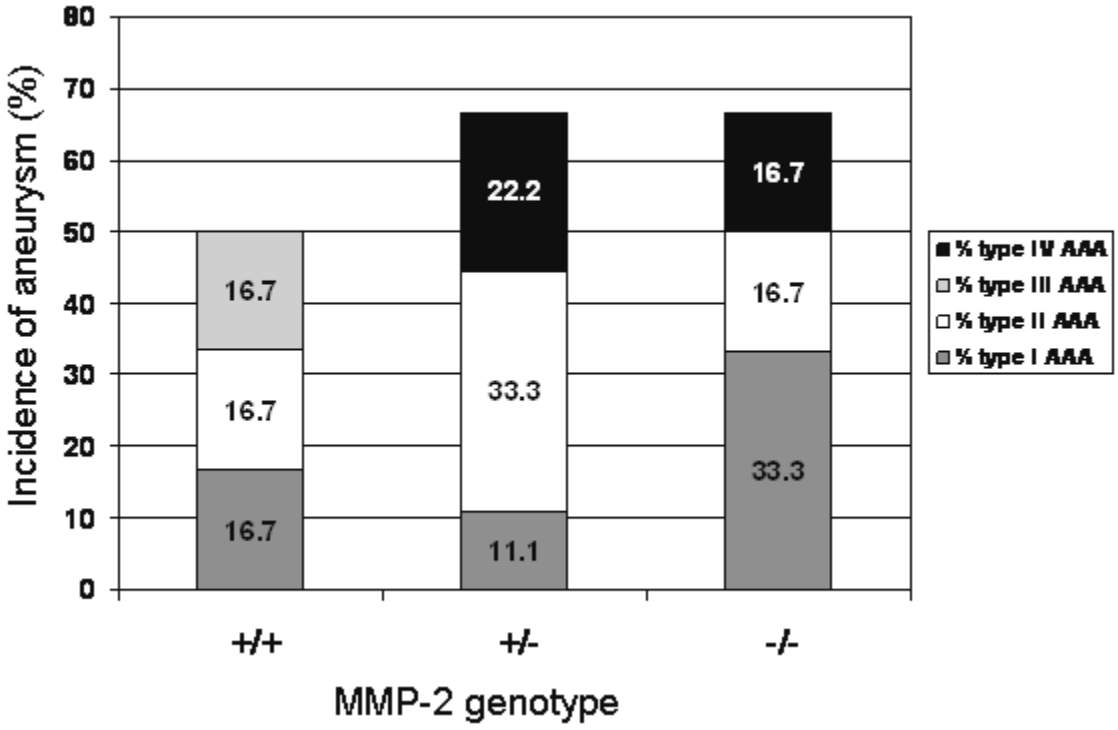


Figure 2.6 Effect of MMP-2 genotype on the incidence of AngII-induced aneurysms in 12-week-old apoE-/- mice Incidence of AAA in mice from each group (n=6-9/group). No significant difference was detected among mice with various MMP-2 genotypes.

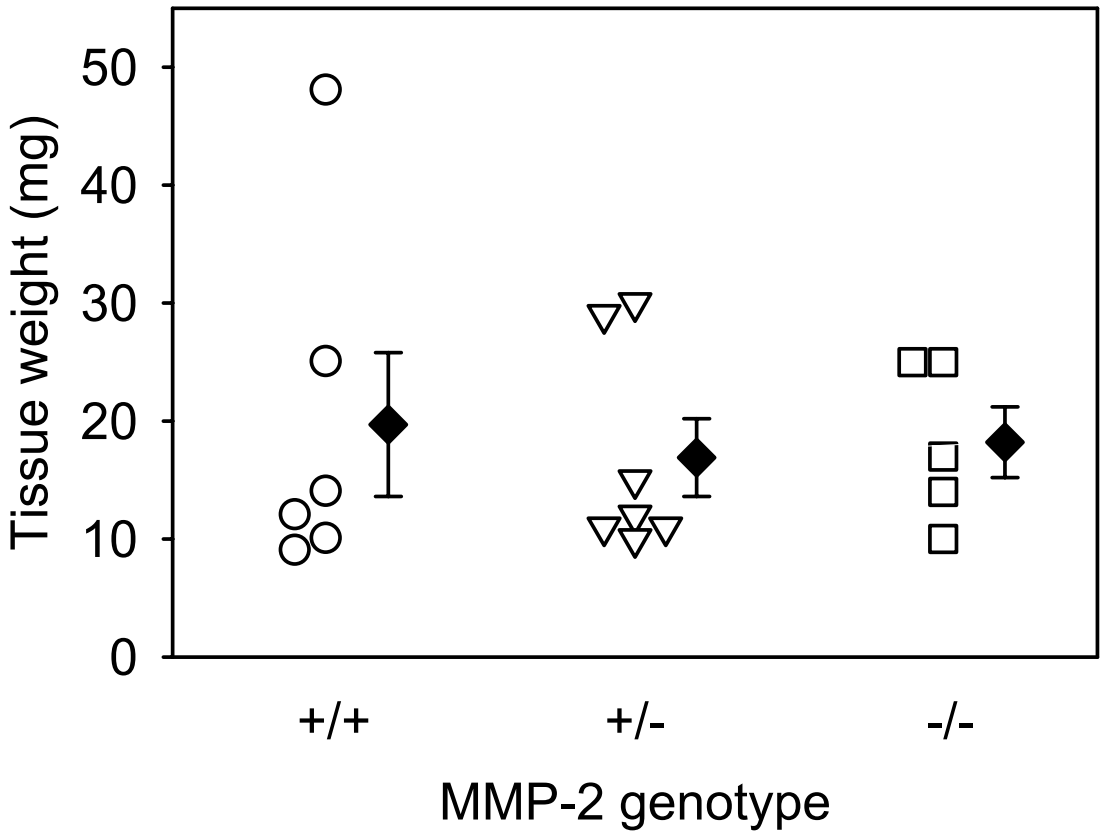


Figure 2.7 Effect of MMP-2 genotype on the severity of AngII-induced aneurysms in 12-week-old apoE-/- mice Abdominal aortas were isolated by cutting the aorta at the level of the diaphragm and the iliac bifurcation. The tissue was weighed to provide an index of AAA severity. No significant difference was detected among mice with various MMP-2 genotypes. Values obtained from individual mice are represented in open symbols, the means are shown as closed symbols and SEM as bars. These data do not include the tissue weight of three mice (two MMP-2+/- mice and one MMP-2-/- mouse) that died from AAA rupture during AngII infusion.

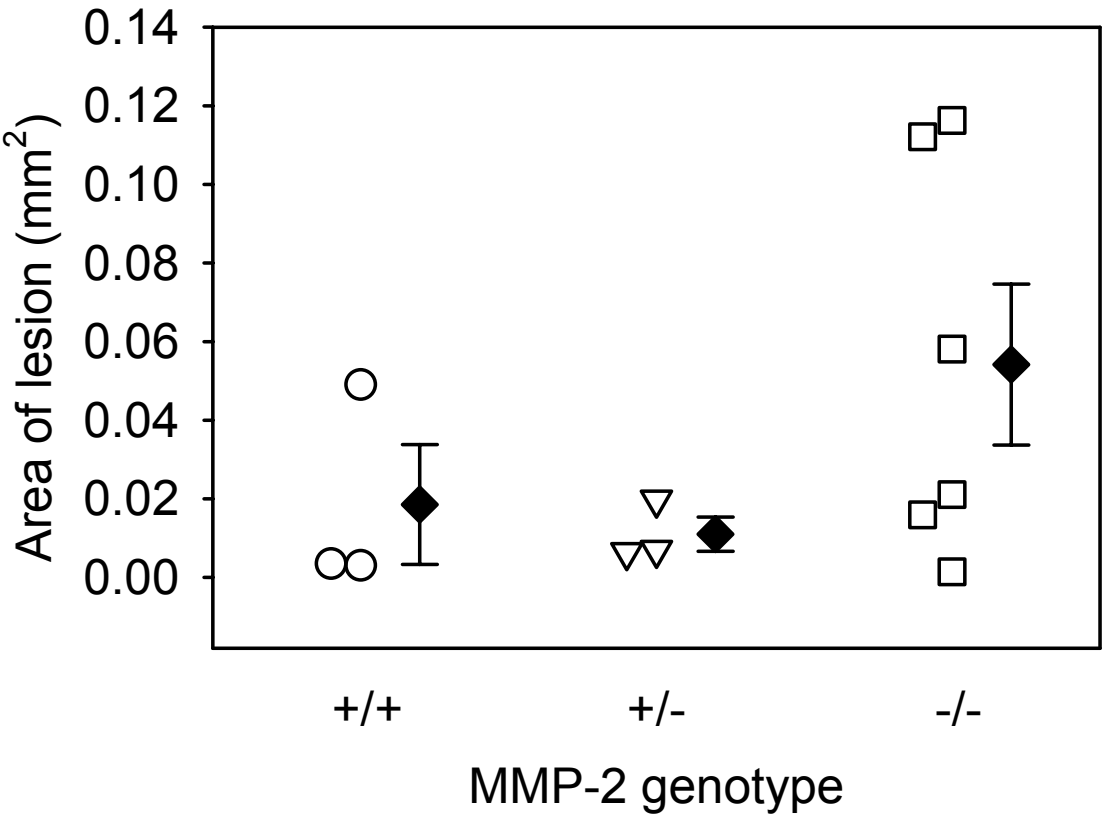


Figure 2.8 Effect of MMP-2 genotype on atherosclerosis in 5-month-old apoE-/-mice Area of atherosclerosis was measured in the sections of aortic root of 5-month-old male mice. Lesion area was determined in 10 μm-thick sections at 80 μm intervals after Oil Red O staining. Values obtained from individual mice are represented in open symbols, the means are depicted as closed symbols and SEM as bars.

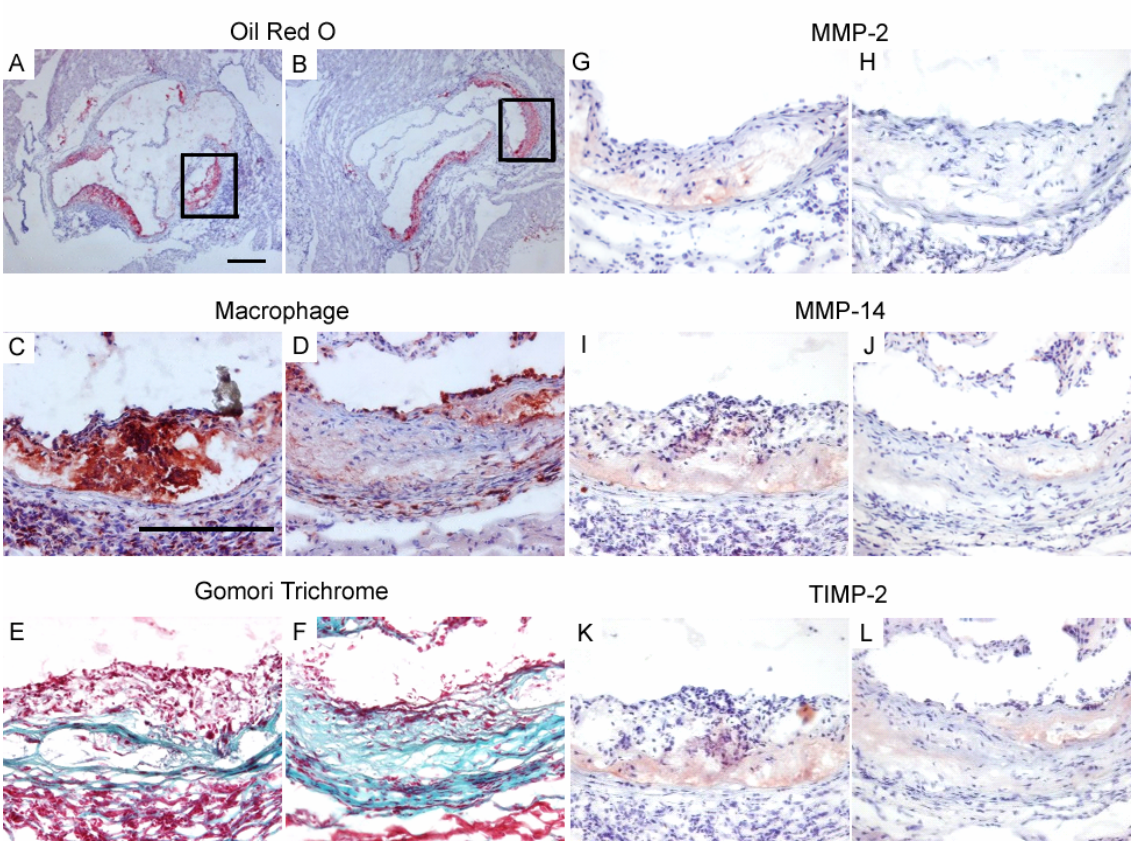


Figure 2.9 Histopathological and immunohistochemical pictures of atherosclerotic lesion on aortic root of 5-month-old apoE-/- mice Cross-sections of the aortic root of 5-month-old male MMP-2+/+ x apoE-/- (A, C, E, G, I, K) and MMP-2-/- x apoE-/- (B, D, F, H, J, L) mice were stained with Oil Red O staining (A and B; 40x), Gomori trichrome staining (C and D; blue-collagen; red-smooth muscle cells). The sequential sections were used to do immunohistochemical staining for macrophages (E) and (F), MMP-2 (G) and (H), MMP-14 (I) and (J), and TIMP-2 (K) and (L). C-F and I-L: the detail of boxed area in the Oil Red O staining in A and B on sequential sections (200 x); Bars indicate 50 μm.

Chapter Three
Phenotypic Changes in MMP-2 Deficient Mice

I. Background

Genetically engineered mice are widely used in the functional analysis of the genes involved in cardiovascular diseases. These mice provide ideal approaches to study the members of gene families that encode several isoenzymes and lack selective inhibitors and inducers. For example, the influence of MMPs in genetically modified mice was investigated in studies as mentioned previously (in Chapter 1). Despite the high level of specificity, sometimes genetic engineering leads to unexpected results. Some of these results may provide insight into new functions of genes, which have not been detected or anticipated based on previous pharmacological interventions.

Frequently, transgenic animals do not show obvious phenotypic changes. The normality of the animal might be due to the insignificance of the gene. However, this normality may also be due to the activation of an adaptive mechanism that effectively compensates for the loss of that specific gene. Therefore, a detailed functional and morphological analysis of the transgenic animal is considered an important issue for the correct evaluation of the gene. It should be kept in mind that in contrast to a pharmacological approach, which usually causes an acute intervention with too little time for an adaptive mechanism, a transgenic approach is more like a chronic intervention allowing the animal time to develop possible compensatory adaptations. [72]

MMP-2 gene mutation in humans

In humans, homoallelic MMP-2 mutation on chromosome 16q12-q22 causes a loss of all three MMP-2 functional domains: the Zn^{2+} binding site catalytic domain, the three fibronectin type II-like domains and the hemopexin

domain. Consequently, MMP-2 activity is completely ablated. MMP-2 ablation is assumed to alleviate the bone absorption in arthritis since MMP-2 is assumed to be a pivotal enzyme for bone and extracellular matrix turnover. However, the paradox in affected individuals is that the absence of MMP-2 leads to an autosomal recessive disease that is characterized by an excessive reabsorption and destruction of bones and arthritis. The patients have a set of 'vanishing bone syndromes', in which the bones, primarily the bones of hands and feet, are severely reabsorbed and destructed. The patients also have chronic arthritis and subcutaneous nodules in the hands and feet. The underlying mechanism is unclear. There are a couple of hypotheses for the findings in these patients – 1)there might be compensatory overproduction of other protease(s) in patients with MMP-2 gene mutant or 2) MMP-2 might be essential for processing of the inductive factor which is required for the activity of osteoblasts or the regulation of the activity of osteoclasts.[134, 188]

MMP-2 gene deficiency in the mouse model

MMP-2 deficient mice were developed in 1998 for use in cancer research. It was soon discovered that MMP-2 reduced angiogenesis and tumor progression in these animals.[97] Although some morphological changes (including a mild growth retardation and delayed mammary gland development) were evident, these mice were not known to mimic the human disease found in bones and joints.[188] It was consistently reported that the inhibition of MMP-2 impairs adipose tissue growth via the formation of a collagen-rich matrix cap in mice.[123] Other phenotypic changes of MMP-2 deficient mice include: 1) a decreased allergen inhaling-induced lung inflammatory cell egression which increases susceptibility to asphyxiation,[35] 2) a significant reduction of endothelial outgrowth, corneal angiogenesis, [103] and extra-retinal neo-vascularization in oxygen-induced retinopathy,[150] and 3) an early onset and severe experimental autoimmune encephalomyelitis due to an increase in MMP-9 expression and activity.[53]

MMP-2 deficient mice were used in research of cardiovascular diseases as well. Some related findings conclude that targeted deletion of MMP-2 attenuates cardiac rupture after experimental myocardial infarction [80,136] and reduces intimal hyperplasia in blood flow cessation in the carotid arteries.[101,114] As mentioned previously in this dissertation, MMP-2 null mice are resistant to $CaCl_2$-induced AAAs.[126] However, there is a shortage of literature investigating characteristics of the vascular wall and inflammatory cells of MMP-2 deficient mice. Such an investigation is essential for an in-depth understanding of the role of MMP-2 in cardiovascular diseases. The surprising result we have obtained in our previous study (in Chapter 2) makes this investigation an even more important event. The genotypic characteristics of several MMP deficient mice are listed in Table 3.1.

II. Materials and Methods

Animals

Six-week-old male mice on C57BL/6 background were obtained from The National Cancer Institute (NCI, Frederick, MD, USA). They were used as control mice to detect the morphological change in aortic wall of MMP-2 deficient mice. MMP-2 deficient mice (C57BL/6 background) were obtained from Dr. Itohara (Brain Science Institute, RIKEN, Saitam, Japan).

ApoE-/- mice on a C57BL/6 background were obtained from the Jackson Laboratory (Bar Harbor, ME, USA). MMP-2-/- mice and apoE-/- mice were then bred and their offspring, generation F1 (MMP-2+/- x apoE+/-), were mated to produce littermates with the desired MMP-2+/+ and MMP-2-/- x apoE-/- mice genotypes (F2). Male MMP-2+/+ and -/- x apoE-/- littermates were used in all subsequent experiments. All of the mice were housed under barrier conditions with normal laboratory diet and water ad libitum. All procedures were performed with the approval of the University of Kentucky Institutional Animal Care and Use Committee.

Elastin staining using Verhoeff's iron hematoxylin

Age and gender-matched MMP-2 -/- and wild type mice on C57BL/6 background were sacrificed and infused with 4% paraformaldehyde via the left ventricle at stable pressure (approximate 100 mmHg) for 60 to 90 minutes. Fragments from the abdominal aorta directly above the renal artery branch were cut and frozen in O.C.T. compound. Sections (8 μm) of frozen aortas were stained in Verhoeff's iron hematoxylin (a mixture of 5% hematoxylin in absolute alcohol (20 ml), 10% Ferric chloride (8 ml) and Verhoeff's iodine (8 ml)) for 20 minutes. Sections were differentiated in 2% ferric chloride. When the nuclei and elastin fibers were still black, and the background was weakly stained, the staining was halted. The sections were washed in distilled water, then in 95% alcohol to remove the iodine coloration. The slides were rinsed in distilled water again and mounted with glycerol gelatin.

Mouse peritoneal macrophage harvest

Age and gender-matched MMP-2 -/- and wild type mice on C57BL/6 background were anesthetized with sterile saline containing 22.7% ketamine 3.4% xylazine, then a cervical dislocation were performed. A slit was made in the outer skin in order to provide a "window" of visibility to the peritoneum. Sterile saline (5 ml) was injected into the peritoneal cavity through the intact abdominal musculature then slowly withdrawn through the same syringe needle. Fluid and cells were transferred into a 15 ml conical tube on ice. After centrifuging at 1,000 x rpm for 5 minutes at RT, the supernatant was discarded. If there were RBCs visually detectable, 5ml of red blood cell (RBC) lysing solution containing 0.83% ammonium chloride and 0.158% Tris HCl (Sigma) was added into the tube. After 2 to 5 minutes, cells were spun down at 1,000 x rpm for 5 minutes. The supernatant was discarded. The cells were lysed again if RBCs were still present. After lysis, RIPA buffer (100 μl) containing 1% NP-40, 0.5% sodium

deoxycholate, 0.1% SDS, 0.001% aprotinin, 1mM sodium orthovanadate, and 0.01% PMSF (phenylmethyl sulfonyl fluoride) was added into each tube. The cells were incubated for 30 minutes on ice then transferred into microfuge tubes. The tubes were centrifuged at 13,000 x g for 10 minutes at 4°C. The supernatant was collected, and stored at -20°C. The protein content was measured using a micro BCA protein assay kit (Pierce, Rockford, IL, USA).

Gelatin zymography

Age and gender-matched MMP-2-/- x apoE-/- and MMP-2+/+ x apoE-/- mice were sacrificed. Frozen aortas (from aortic arch to the iliac bifurcation) were homogenized. Gelatin zymography was performed as described previously in Chapter 2. In this study, we compared the expression of MMP-9 in aortic extracts of these mice. Age and gender-matched MMP-2 -/- and wild type mice on C57BL/6 background were sacrificed and peritoneal macrophages were isolated as described above. Gelatin zymography was performed to compare the expression of MMP-9 in peritoneal macrophages. The MMP-9 level in aorta extracts of these mice was measured as well.

Peripheral white blood cell counting

Age and gender-matched MMP-2 -/- and wild type mice on C57BL/6 background were sacrificed. The experiment was performed as described previously (in Chapter 2).

Statistics

Student's t-test was used to test the difference between two groups. All of the statistical analyses as above were done by a SigmaStat 2.03 software. Values with $P < 0.05$ were considered statistically significant.

III. Results

MMP-2-/- mice have lower body weights

Age-matched male MMP-2 deficient mice have a lower body weight as compared to MMP-2 wild type mice (n = 7/group; $P < 0.01$). Corresponding to the lower body weight, the weight of the spleens, livers and hearts of MMP-2 -/- mice were lower as well. However, no difference was detected in the heart/body weight and liver/body weight ratios of MMP-2+/+ and -/- mice (Table 3.2). We also observed that MMP-2 deficient mice have flatter faces and noses (Figure 3.1), as well as shorter tails than their wild type counterparts. Although MMP-2 is involved in ovulation, the reproductive ability of MMP-2 mice appears normal (8-10 pups per litter).

MMP-2-/- mice have a higher peripheral WBC count

Blood was collected from mice (n = 10 -11/group). The MMP-2 genotype did not create any significant differences in the number of red blood cell, platelets, or concentration of hemoglobin. Interestingly, the number of WBC was dramatically raised in MMP-2-/- mice ($12.2 \pm 1.5 \times 10^3$ cell/mm^3) when compared with MMP-2+/+ mice ($6.5 \pm 0.6 \times 10^3$ cell/mm^3; $P < 0.05$; Figure 3.2).

MMP-2-/- mice have increased branching of the elastin fibers in the abdominal region of the aorta

Sections of abdominal aorta from six-month-old wild type and MMP-2-/- male mice were stained with Verhoeff's iron hematoxylin (n = 9-10/group). Branches of elastin fibers in the aortic walls were counted. An increased level of branching was observed in aortas from MMP-2-/- mice compared with aortas from MMP-2+/+ mice. (19.44 ± 1.07/section vs. 6.9 ± 1.13 per section; $P < 0.05$;

Figure 3.3)

MMP-2-/- x apoE-/- mice have increased MMP-9 levels in aortic extracts

Gelatin zymography of aortic extracts from age and gender matched MMP-2+/+ and MMP-2-/- x apoE-/- mice was performed. The average band intensity from gelatin lysis by MMP-9 was increased 143% in the extracts of aortas of MMP-2 deficient mice compared with the aortas of MMP-2 +/+ (P < 0.01; Figure 3.4). We also sought to determine the MMP-9 level in MMP-2-/- and MMP-2+/+ mice on a C57BL/6 background (apoE+/+). However, the expression of MMP-9 in these aortas was too low to be detected by gelatin zymography (data not shown).

MMP-9 expression tends to increase in MMP-2-/- peritoneal macrophages

Gelatin zymography of peritoneal macrophage extracts from age-matched male MMP-2 wild type and MMP-2 deficient mice was performed. The average intensity of bands of gelatin lysis by MMP-9 was increased 98% in the extracts of aortas of MMP-2 deficient mice compared with aortas of MMP-2 wild type mice. However, there was no statistical significance (P = 0.2; Figure 3.5).

IV. Discussion

MMP-2 null mice show some phenotypic differences when compared to their MMP-2 wild type counterparts, although these differences are less dramatic than those associated with MMP-14 null mice, which have severe bone and connective tissue abnormalities, inadequate collagen turnover and early postnatal death [85] (Table 3.1).

In our current study, we observed that MMP-2 deficient mice have lower body weight, flatter faces (noses) and shorter tails than their MMP-2 wild type counterparts. This might be due to a mild abnormality in the development of

connective tissues, such as bone, cartilage, and adipose tissue. We were surprised to observe that MMP-2 deficient mice exhibited a similar size of atherosclerotic lesion and a similar incidence of AngII-induced AAA in a hyperlipidemic mouse model (see Chapter 2). Therefore, we did several experiments to determine the underlying mechanisms.

We confirmed the genotype of MMP-2 deficiency in the knockout mice by gelatin zymography. The ablation of the MMP-2 phenotype allowed us to concentrate on the peripheral pathways. We measured blood pressure to examine the hemodynamic conditions present in the MMP-2 deficient mouse. Pressures for both knockout and control mice under both basal conditions and AngII infusion were consistent with previous findings. We then compared the aortic wall structure of MMP-2 -/- and +/+ mice. Interestingly, we observed an increased number of branches in elastin fibers of the aorta of MMP-2 null mice compared with aorta of wild type mice. This may provide protection from AAAs by strengthening the vascular wall. It also may signal the destruction and remodeling of ECM, and increased rigidity of vascular wall. Future study is required to measure the vascular contractility of MMP-2-/- mice.

MMP-2-/- x apoE-/- mice consistently expressed elevated levels of MMP-9 in aortic tissue extracts as compared to wild type controls, as determined by gelatin zymography. However, in aortas of MMP-2-/- mice on a C57BL/6 wild type background (apoE+/+) and MMP-2 wild type control mice, the MMP-9 level was too low to be detected by gelatin zymography (data not shown). This indicates that hyperlipidemia might be involved in the compensatory overexpression of MMP-9 in apoE-/- mice. Another potential mechanism underlying this compensation is that MMP-2 might be required to regulate the expression and/or activation of other elastinases. Recently, it was reported that the MT1-MMP/TIMP-2/MMP-2 complex, in addition to facilitating the activation of pro-MMP-2, could be involved in regulating the expression of MMP-9 in lymphocytes of mice.[53] MMP-2 deficient mice showed a tendency to have higher MMP-9 expression in peritoneal macrophages as compared to wild type control mice. However, there was not a statistical significance.

In addition, we detected a significant increase in peripheral white blood cell (WBC) number in MMP-2 null mice. Infection, leukemia and tissue damage are the three most common reasons for a high WBC count and secondary immunodeficiency. However, splenomegaly, which is an important sign of infection, is not observed in MMP-2 deficient mice. One study has been published that studied the role of MMP-2 expression in clinical course of acute myeloid leukemia (AML). It surprisingly showed that 82% of the MMP-2 positive patients survived for over 3 years, whereas all MMP-2 negative patients relapsed within 13.5 months of diagnosis.[113] Compared to solid tumors, this finding is surprising, as increased MMP-2 expression in solid tumors is often related with a more biological aggressive and invasive phenotype.[41,81,142] However, the role of MMPs in increased angiogenesis and leukemic cell growth have not been extensively investigated. Further studies need to be completed to determine the mechanism underlying the high peripheral WBC number of MMP-2 null mice.

Our studies identified phenotypic changes in the aortic vessel wall and peripheral white blood cells of MMP-2 null mice. Many of these changes are currently unexplained and further investigation may bring insight into the broader biological role of MMP-2. These data support that MMP-2 plays a complex role beyond degradation of ECM in vascular diseases.

Table 3.1 Phenotypes of transgenic mice lacking MMP-2, MMP-9, MMP-14 and TIMP-2 genes

Gene deficiency	Phenotypes
MMP-2	Mild growth retardation and delayed mammary gland development [190] reduced intimal hyperplasia in a mouse carotid artery blood flow cessation model [101,114] Reduced AAA formation induced by abluminal application of calcium chloride in mice[10]
MMP-9	Resistant to aneurysms induced by elastinase infusion and abluminal application of $CaCl_2$[10,162] Prolonged contact dermatitis[199] Defective osteoclast recruitment [52]
MMP-14	Reduced collagen turnover Impaired endochondral ossification and angiogenesis; Death before the onset of sexual maturity[83,222] Severe postnatal growth-retardation and skeletal abnormalities[212]
TIMP-2	Deficient MMP-2 activation[212]

Table 3.2 Body and organ weight of 6-week-old male MMP-2+/+ and -/- mice

Mouse	MMP-2 +/+	MMP-2 -/-
Body weight (g)	22.4 ± 0.6	18.4 ± 0.9*
Spleen weight ($x10^{-2}$ g)	7.9 ± 0.3	6.7 ± 0.5*
Spleen/body weight (%)	0.35 ± 0.01	0.36 ± 0.02
Liver weight (g)	1.30 ± 0.04	0.98 ± 0.10*
Liver/body weight (%)	5.8 ± 0.2	5.3 ± 0.4

Data represent mean ± SEM for groups of 7 mice.
*P<0.05 with respect to MMP-2 +/+ mice

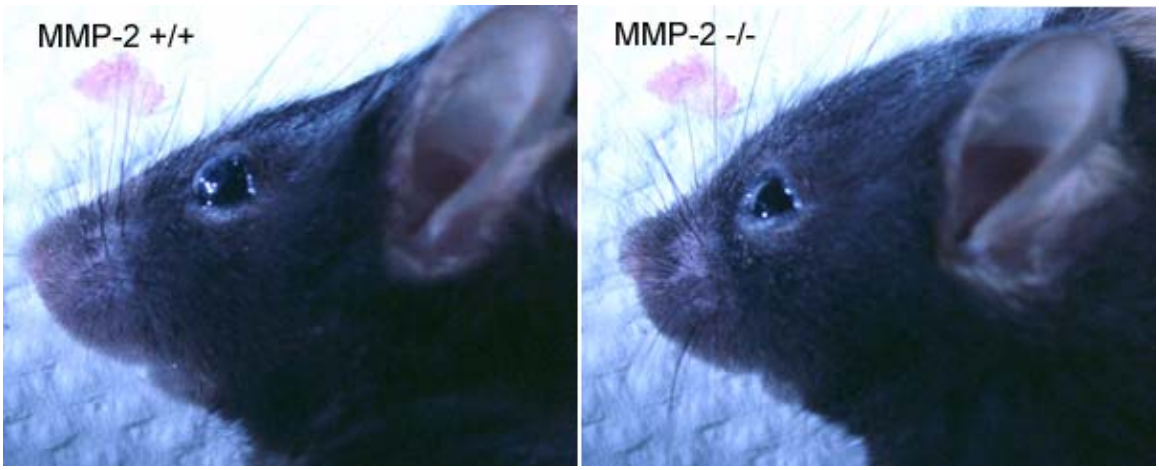


Figure 3.1 Craniofacial differences between MMP-2 +/+ and MMP-2 -/- mice. Representative pictures of a MMP-2+/+ and -/- mouse were taken while subjects were anaesthetized. The MMP-2 deficient mouse (on the right) has a depressed face and shorter nose than the MMP-2 wild type mouse.

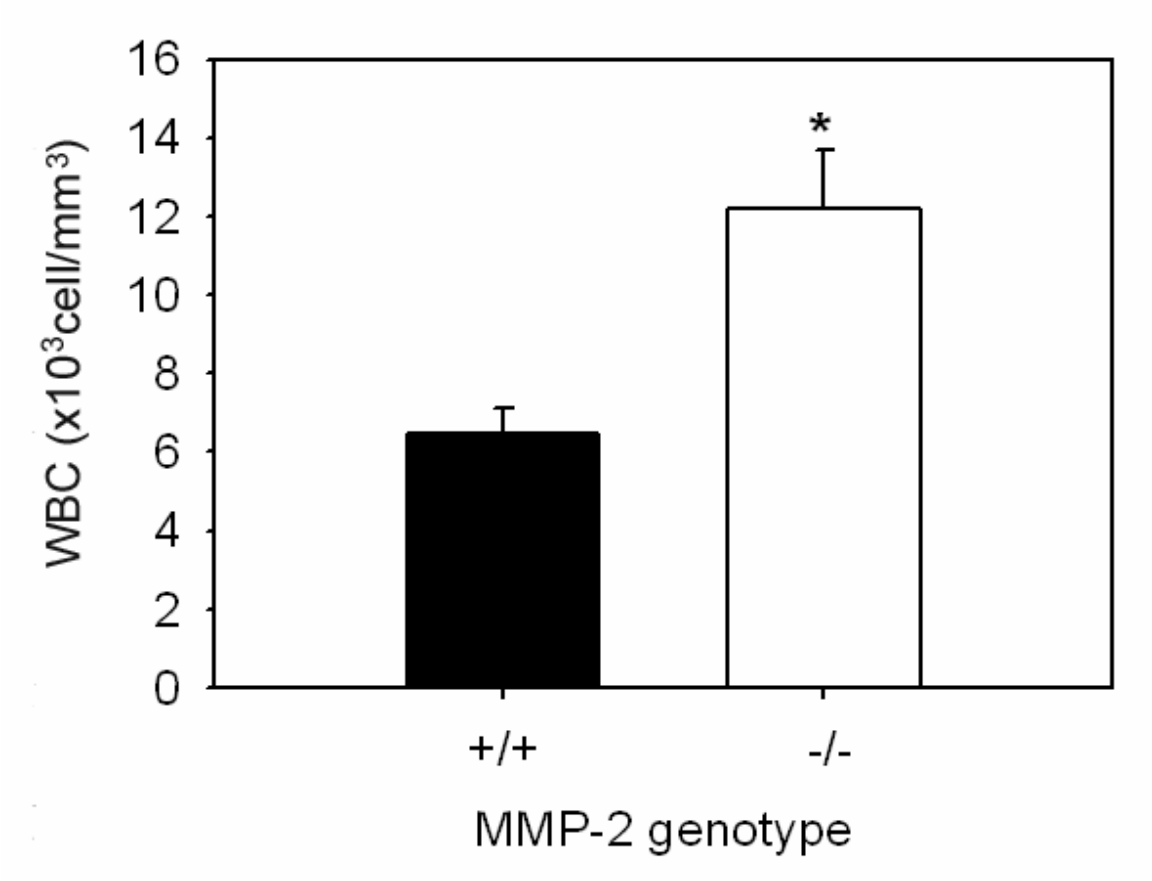


Figure 3.2 Peripheral WBC counts from MMP-2 -/- and MMP-2 +/+ mice. Blood was obtained by retro-orbital bleeding and the cells were counted using a Coulter counter (n=10-11/group). Histobars represent the means and bars are SEM. (*P < 0.01 for comparisons of MMP-2 deficient mice versus wild type mice)

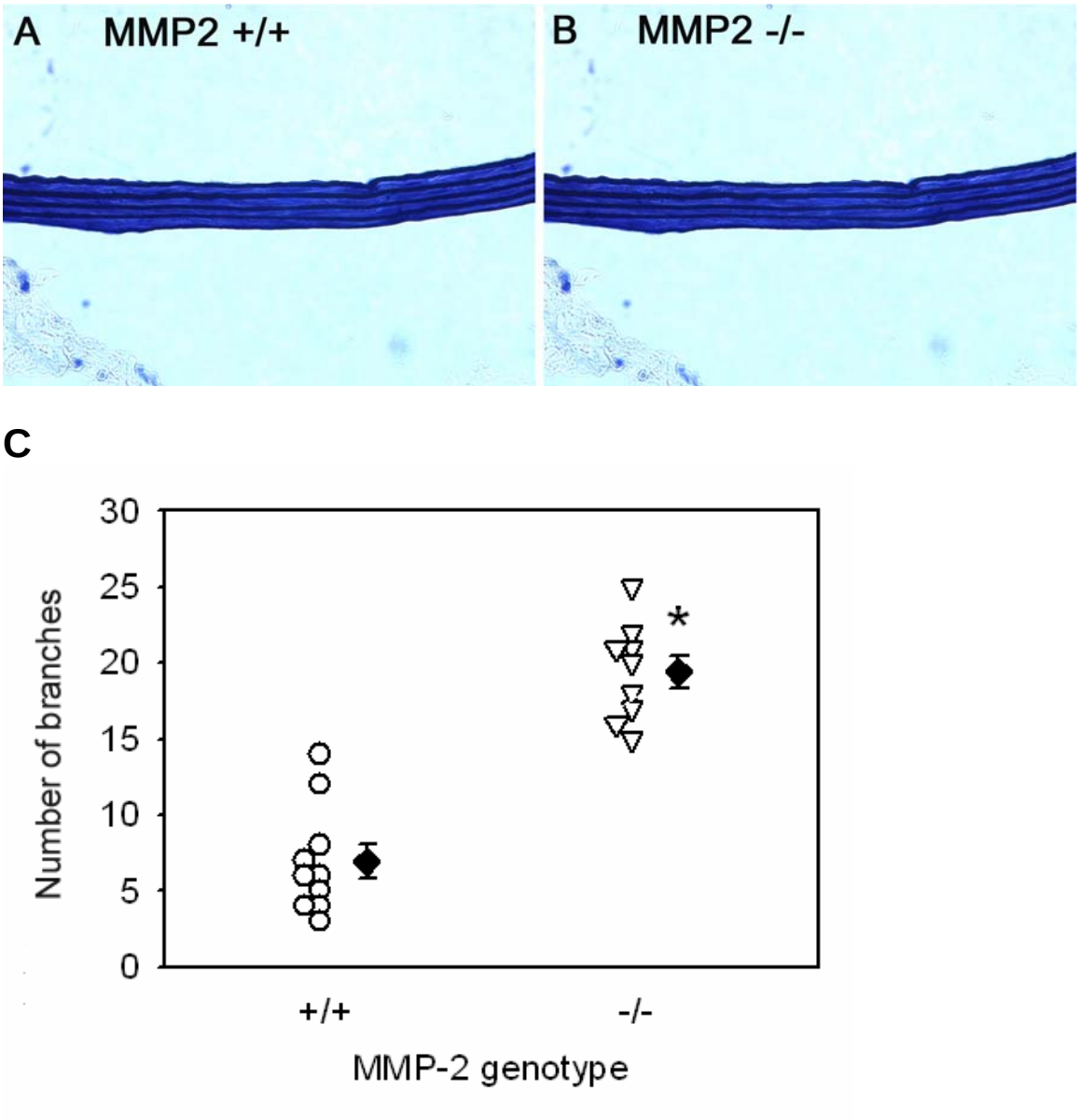


Figure 3.3 Morphological changes in the aortic wall of MMP-2 -/- and MMP-2 +/+ mice (A and B) Representative sections of elastin staining using Verhoeff's iron hematoxylin of abdominal aortas from six-month-old male MMP-2+/+ and -/-. Arrows indicate branches of elastin fibers. (C) The number of elastin fiber branches counted in each section. Values obtained from individual mice are represented as open circles, means are depicted as closed diamonds and SEM as bars. (n = 9-10/group; *P < 0.05 for comparisons of MMP2+/+ versus -/- mice)

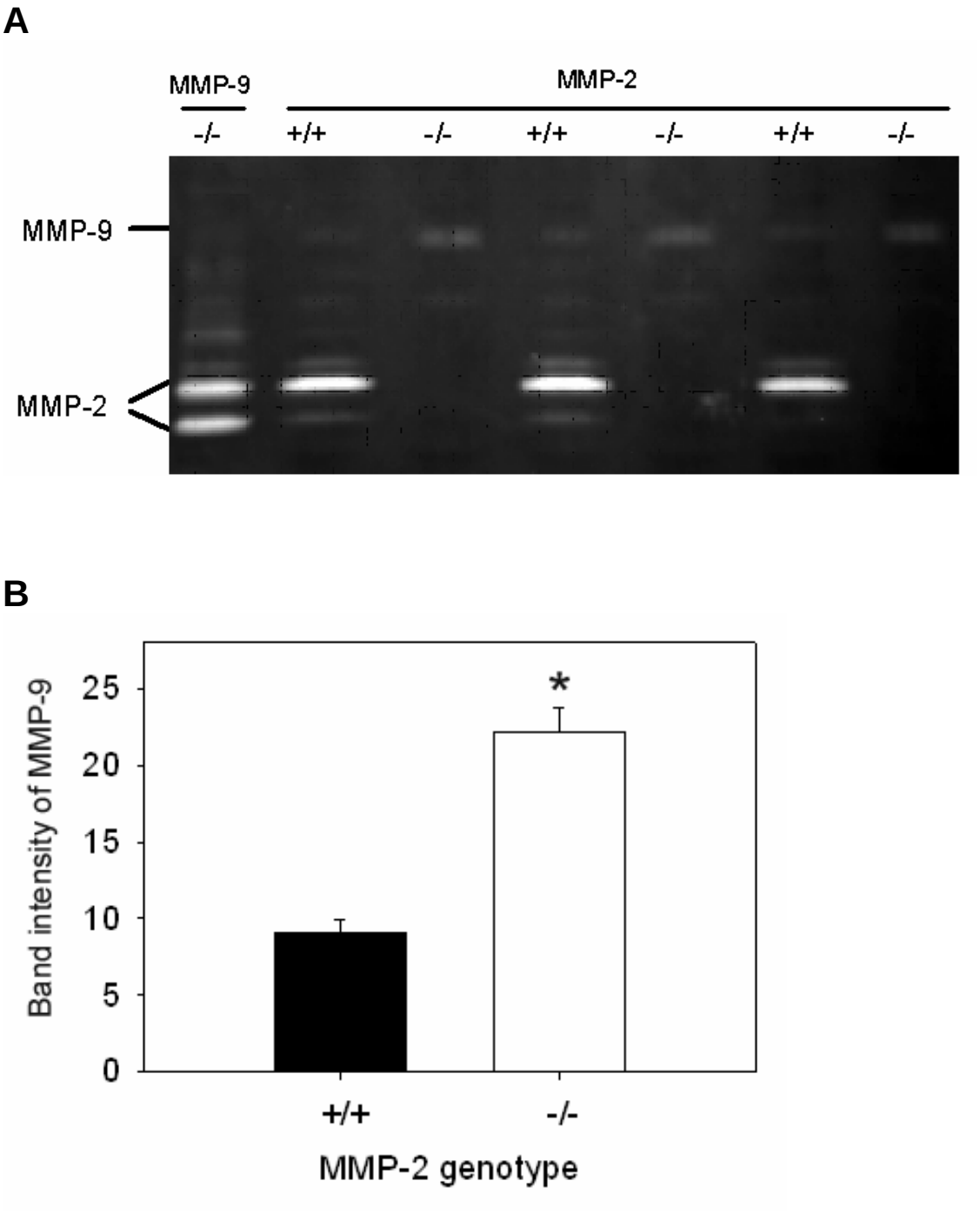


Figure 3.4 Aortic extract expression of MMP-9 in MMP-2-/- and MMP-2+/+ x apoE-/- mice. (A) Gelatin zymography of protein extracts from mouse aortas. (B) Gelatinase activity was quantified by densitometry. Data were obtained from 3 individual mice. Histobars represent the means and bars are SEM. (*P < 0.05 for comparison of MMP-2 deficient versus MMP-2 wild type aortas)

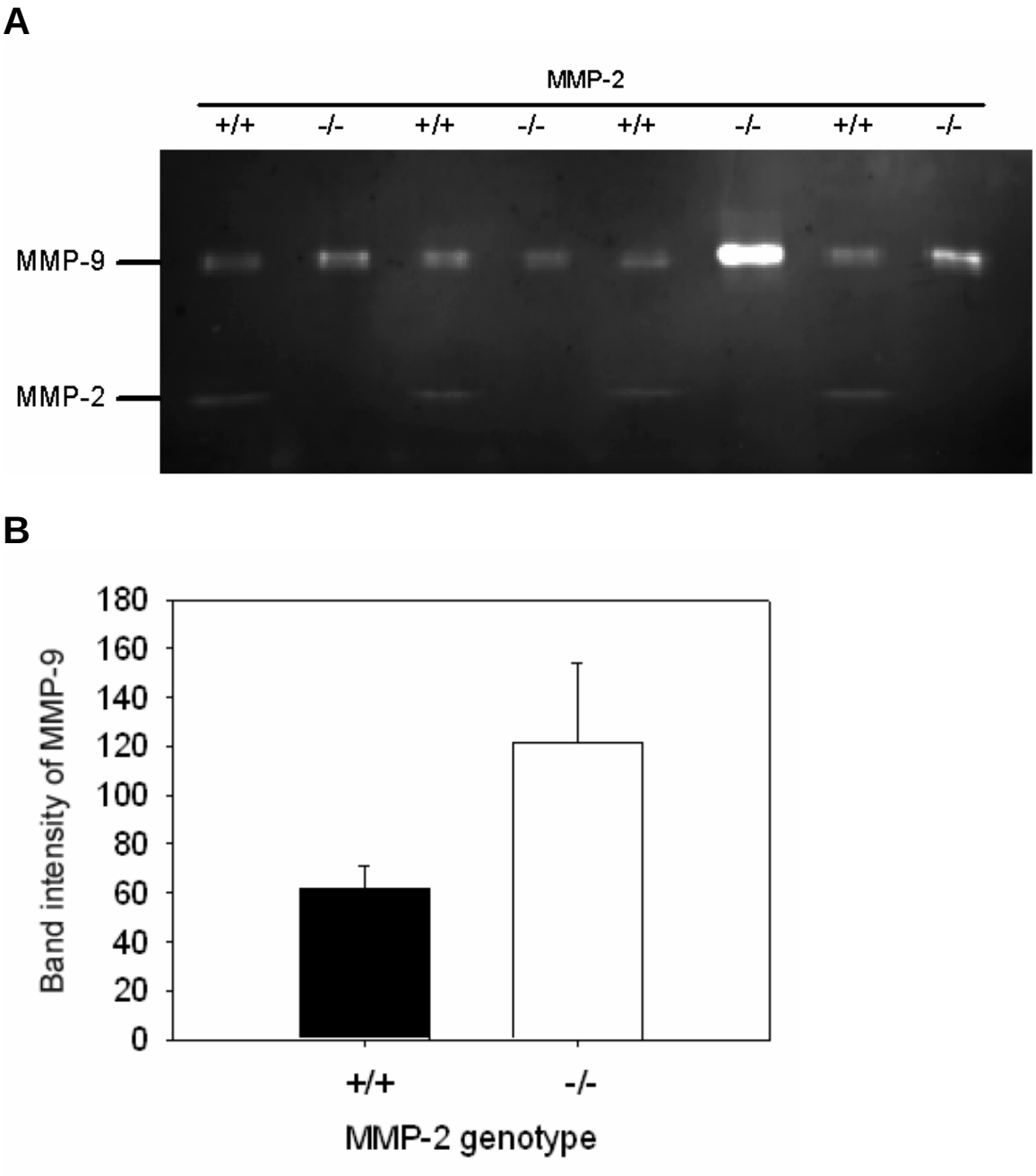


Figure 3.5 Mouse peritoneal macrophage expression of MMP-9 in MMP-2 -/- and MMP-2 +/+ x apoE-/- mice. (A) Gelatin zymography of protein extracts of macrophages. Protein extracts (15µg in each line) from aortas of MMP-2 wild type or MMP-2 deficient apoE-/- mice. (B) Gelatinase activity was quantified by densitometry. Data were obtained from 4 individual mice. However, there was not a statistical significance (*P* = 0.2). Histobars represent the means and bars are SEM.

Chapter Four

Reduced Adhesion Ability and Integrin Beta 3 Expression in MMP-2 Deficient Macrophages (*in vitro*)

I. Background

The recruitment of inflammatory cells from the circulation and their trans-endothelial migration into the vascular wall are prominent features in both atherosclerosis and AAAs. There are considerable new insights into the mechanism underlying the recruitment of inflammatory cells. The initiating step of this process might be the random contact of inflammatory cells to the endothelium, followed by cell-rolling, -tethering, -arresting, and cell-transmigrating through the endothelium. Adhesion molecules/receptors, including selectins, immunoglobin-like molecules (such as ICAM, VCAM, and PECAM), cadherins, and integrins are involved in this process. [68, 93, 94]

As mentioned previously, many types of cells in the vascular wall including endothelial cells, VSMCs, and fibroblasts, produce MMP-2 constitutively. Inflammatory cells, such as macrophages, T lymphocytes, and mast cells, produce MMP-2 as well. Unlike the mesenchymal cells, inflammatory cells produce MMP-2 in a relative small amount. During the occurrence of inflammation, including vascular diseases, pathophysiological changes might modify the production of MMP-2 by inflammatory cells. For instance, secretion of MMP-2 by T lymphocytes can be upregulated by an interaction between the cell and fibronectin through alpha 4 beta 1 integrins, as well as a ligation of VCAM-1.[216] The expression of macrophage-derived MMP-2, which is not as abundant as macrophage-derived MMP-9, was significantly reduced in CCR-2 deficient mice.[153] In cultured rat peritoneal macrophages, an increased MMP-2 expression and activity can be elicited by AngII and subsequently prevented by losartan, an AT1 receptor antagonist.[121] As mentioned in Chapter 1, most MMPs are secreted

as zymogens and are not activated spontaneously. It is thought that MMPs secreted by macrophages could serve as activators of MMPs derived from other cell types, including VSMCs.[147]

The potential role of MMP-2 in the regulation of macrophage behavior and cell-matrix interaction

MMP-2 activity might influence the behavior of inflammatory cells, playing a pivotal role in cardiovascular diseases. There is evidence that MMP-2 is able to facilitate neutrophil-endothelial cell adhesion by cleaving pro-endothelin into an active form in vitro. [58] Furthermore, it was reported that degradation fragments of ECM components, generated by the action of MMP-2, are involved in macrophage migration in a myocardial infarction mouse model.[136] As mentioned in Chapter 1, ECM proteins may bind to cytokine receptors, growth factor receptors and cell-adhesion receptors thus transducing receptor-mediated signalings, which are important for regulating biological functions of the cell, including mobility, proliferation, and apoptosis. Meanwhile, the binding of ECM proteins may influence cell behavior by influencing the binding of other ligands to these receptors.

Integrins are the major receptors mediating cell-to-ECM and cell-to-cell adhesion. They have been studied extensively, becoming the most-understood cell adhesion receptors.[47] The integrin family is comprised of 24 members. Each member is categorized as a heterodimeric transmembrane glycoprotein, containing one of 18 alpha and one of 8 beta subunits. Differentiation is achieved by the unique, non-covalent coupling of these subunits. Integrins mediate cell adhesion by activating a variety of protein tyrosine kinases like focal adhesion kinase (FAK) and Src-family kinases. Integrins also initiate intracellular signaling molecules which control cell shape, migration, proliferation, differentiation and survival.[68] In adherent cells, integrins are apparently in a constitutively active form which is induced by inside-out signaling events such as Rho-like GTPase, certain cations (e.g. Mn^{2+}), and antibodies (Figure 4.1). This ligand binding at the

cytoplasmic domain of integrins changes their conformation from inactive to active form by splitting away the alpha subunit, which allows the interaction of beta subunit with the cyto-skeletal components. In the active conformation, the affinity of integrin and its ligands is enhanced, therefore, the outside-in signaling pathway can be turned on and modulate cell behaviors. [36,48,88] Although the mechanism underlying the regulation of integrin-mediated cell adhesion is unclear, it is clear that integrin-mediated cell spreading leads to increased adhesion.

Cell migration is a process that also requires the strict regulation of integrin-mediated adhesion and release of the adhesive cell. A net movement of the cell is due to the cooperation of two elements: 1) the repeated cycle of the assembly and disassembly of adhesive complexes which leads to the protrusion of the cell membrane at the front part and 2) the cyto-skeletal contraction and detachment of the cell rear part. There is evidence that a pool of integrins is internalized and recycled to the rear part of the cell, demonstrated by rear-ligand-bound integrins left behind on the surface of substrate. [17,155,168] Furthermore, the interaction between integrins might affect cell migration and surface expression levels of these molecules, causing a “clustering” of integrins, which may influence the affinity of both integrins and their ligands.[205] The interactions between integrins and receptor kinases such as the EGF and the PDGF receptors have also been reported. These interactions could lead to the clustering of growth factor receptors, thereby enhancing the downstream-signalings.[60]

Like other integrins, integrin alpha v beta 3**,** is involved in cell adhesion, migration and survival.[19,175,204] As mentioned in Chapter 1, alpha v beta 3 integrin also functions as an anchor protein of MMP-2 on the cell surface, becoming involved in MMP-2 activation. Integrin alpha v beta 3 is expressed by many cell types, including macrophages, endothelial, smooth muscle, fibroblasts, osteoclasts, and others.[133] It associates with a variety of ECM proteins such as vitronectin, osteopontin, fibronectin, fibrinogen, thrombospondin, proteolysed collagen, and von Willebrand factor. It also associates with some important cell

surface molecules including MMP-14, MMP-2, VEGFR2, IAP, and CD47.[46,82,86,187,212] The expression of integrin alpha v beta 3 on endothelial cells was found to increase when nitric oxide (NO) levels were increased in rat cardiac fibroblasts. Related to this, the blocking antibodies to beta 3 attenuated AngII-induced adhesion in rat cardiac fibroblasts.[104,116] Another study reported that blocking integrin alpha v beta 3 inhibits macrophage adhesion and migration.[214] Pharmacological blockade of the integrin alpha v beta 3 inhibits angiogenesis in cancer and rheumatoid arthritis patients.[23] Furthermore, an organic molecule (TSRI265) which selectively blocks integrin alpha v beta 3 interaction with MMP-2 without influencing MMP-2 activity, inhibits angiogenesis and tumor growth in nude mice.[19,184] It was also reported that alpha v beta 3 integrin regulated macrophage foam cell formation, possibly influencing the progression of atherosclerosis in humans.[6]

In this study, we sought to investigate the influence of MMP-2 deficiency on certain cell behaviors found in mouse peritoneal macrophages. We also sought to examine the potential effects of the interaction between MMP-2 and integrin alpha v beta 3 on macrophage behavior.

II. Materials and Methods

Adhesion assay of peritoneal macrophages

Peritoneal macrophages were collected from six to eight-week-old MMP-2+/+ and MMP-2-/- male mice as described previously (see Chapter 3) and plated (4 x 10^5 cells/well) in collagen I-coated 24-well plates (Becton Dickinson, Bedford, MA, USA). Cells were incubated in serum free DMEM media (800 μl/well) at 37 ^oC for 30 minutes in a humidified incubator containing 95% air and 5% CO_2. The MPMs were then washed 3 times with serum free DMEM media. Attached cells were either stained with hematoxylin for cell counting, or harvested and lysed to measure the protein content using a micro BCA protein assay kit as described previously (Chapter 3). Cell counting was performed using

ImagePro software. Six independent fields from each well (5 wells for each group) were analyzed using a light microscope at a magnification of 100X.

For controls, peritoneal macrophages from 6-week-old C57BL/6 mice were harvested and incubated with 10μM TSRI265 (Calbiochem, San Diego, CA) in DMEM containing 10% serum replacement (heat-treated bovine albumin and insulin, without MMP-2 and 9; Sigma) at 37 ^{o}C for 3 hours in a humidified incubator containing 95% air and 5% CO_2. This compound is a known disruptor of MMP-2 binding to integrin alpha v beta 3 without influencing MMP-2 activation. After three washings with serum free media, the cells were harvested and lysed for protein content, measured using a micro BCA protein assay kit as described previously.

Invasion assay of peritoneal macrophages

Peritoneal macrophages were collected from six to eight-week-old wild type mice as described previously (Chapter 3). A twenty four-well matrigel invasion chamber (BD Biosciences, San Diego, CA, USA) was used. The upper chambers contained a matrigel layer and a microporous filter (8 μm pore diameter). Either no serum or 10% FBS in DMEM 750μl was added in the bottom chambers. Macrophages (4 x 10^5/well) were seeded on the upper chamber in 500μl of DMEM containing 10% serum replacement either with or without TSRI265 (10μM) and incubated at 37 ^{o}C for 18 hours in a humidified incubator containing 95% air and 5% CO_2. The cells that migrated to the lower chamber were fixed and stained with hematoxylin and counted as described above.

RT-PCR

mRNA was isolated from peritoneal macrophages using a mRNA isolation kit (Promega, Madison, WI, USA). The primer sequences of integrin beta 3 are: forward 5'-GGGGACTGCCTGTGTGACTC-3 and reverse 5' CTTTTCGGTCGTGGATGGTG-3'.[15] The conditions of the cycling reactions are

as follows: 40 cycles of denaturation at 94°C for 30 seconds, annealing at 58.1°C for 1minute, extension at 68°C for 2 minutes, followed by 1 cycle of elongation at 68°C for 2 minutes. The mRNA signals of beta actin were used as loading controls. The primer sequences of beta actin are: forward 5'-GAGACCTTCAACACCCC-3' and reverse 5'-GTGGTGGTGAAGCTGTAG-3'.[39] The conditions of cycling reactions are: 40 cycles of denaturation at 94°C for 30 seconds, annealing at 60°C for 1minute, extension at 68°C for 2 minutes, followed by 1 cycle of elongation at 68°C for 2 minutes.

Immunofluorescence staining for integrin beta3 (CD61) in MPMs

Peritoneal macrophages of MMP-2 deficient and wild type mice were cultured in an 8-well Lab-Tek chamber slide (0.3 x 10^6/well) in serum free DMEM for 3 hours. The media was deleted and the cells were fixed in cold acetone for 10 minutes. After fixation, the cells were washed twice with 1x PBS. Cells were incubated with 15% goat serum for 3 hours at room temperature or overnight at 4º C to block the non-specific reaction, then incubated with purified hamster anti-mouse CD61 (integrin beta3) antibody (1: 20; 2C9.G2; BD Pharmingen, San Diego, CA, USA) overnight at 4°C. After washing 5 times with 1x PBS, the cells were incubated with Alexa Fluor 488 conjugated goat anti-hamster secondary antibody (1: 500; A-21110; Molecular Probes, Eugene, OR, USA) for 30 minutes at RT. The cells were washed 8 times with 1xPBS. The genomic nuclei were counterstained with Hoechst 33258 (1µg/ml) for 5-10 minutes. The chamber was removed. The slides were mounted with gel/mount (Catalog number M01, Biomed, Foster City, CA, USA). Images were captured digitally by a fluorescence microscope (Nikon eclipse E600, Japan) with exposure times kept constant.

Flow cytometry of integrin beta 3 for MPMs

Mouse peritoneal macrophages (1x10^6 cells/ml/ FACS tube) were incubated with purified rat anti-mouse CD16/CD32 monoclonal antibody (1:100,

30µl/0.02µg/10^6 cells; BD Pharmingen) in 1mM EDTA containing Hank's media or 1xPBS/0.5% BSA for 15-30 minutes at 4°C. This reduces the Fc receptor-mediated binding by antibodies of interest. The cells were incubated with R-Phycoerythrin (R-PE)-conjugated rat anti-mouse CD11b (1:20, 2µl/0.02µg/10^6 cells) and fluorescein isothiocyanate (FITC)-conjugated hamster anti-mouse CD61 (2µl /1µg/10^6 cells) monoclonal antibody (BD Pharmingen) for 30 minutes on a rocker at 4°C. Cells were then centrifuged at 1,000x rpm for 5 minutes at 4° C and the supernatant aspirated. After washing twice with 2.0 ml washing buffer (1xPBS/0.5% BSA), the cells were re-suspended in 250µl sterile FACS tubes with 1 x PBS solution. Immunofluorescence was analyzed with a flow cytometer.

Statistics

Data are presented as means ± SEM. One-way ANOVA was performed to test the equality of three or more means with one independent variable at one time by using variances. Student's t-test was used to test the difference between two groups. All of the statistical analyses above were done with SigmaStat 2.03 software. *P* values < 0.05 were considered statistically significant.

III. Results

Disruption in the binding of MMP-2 to integrin alpha v beta 3 reduces peritoneal macrophage invasion but not adhesion

We sought to determine the influence of the interaction between MMP-2 and integrin alpha v beta 3 mouse peritoneal macrophages on the certain cell behavior, particularly adhesion and invasion. Application of TSRI265, a disruptor of MMP-2 binding to integrin alpha v beta 3, led to a 53 percent reduction in macrophage invasion through matrigel when compared to the non-treated cells (*P* < 0.01; Figure 4.2). The lower chambers, containing serum free media, were used as controls. The protein amounts from attached cells were also measured

after incubating with TSRI265 (10μM) for 3 hours and washing. However, no significant change in cell adhesion was detected (P = 0.106; Figure 4.3).

Peritoneal macrophages from MMP-2 -/- mice have reduced adhesion activity

In this experiment, we sought to examine the migration activity of peritoneal macrophages of MMP-2 deficient mice. The cell adhesion assay was performed. We observed a reduction in cell migration that might be due to the reduced adhesion ability of MMP-2 deficient macrophages. Peritoneal macrophages were collected from eight-week-old male MMP-2+/+ and -/- mice (n = 3-5/group). After culturing (30 minutes) and washing, the attached cell number of MMP-2-/- macrophages was significantly less compared to the cell number of wild type macrophages (51.8 ± 16.3/well vs.770.2 ± 126.5/well; P < 0.001). To confirm this data, after washing, the attached cells were collected and lysed. The protein content of the cell lysate was measured. Correspondingly, we observed that the protein concentration from the cell lysate of MMP-2 deficient macrophages was 57% lower than the protein concentration from wild type macrophage cultures (P < 0.001). The experiment was repeated without culturing or washing, and the protein content of wild type and MMP-2-/- macrophages was found to be similar (Figure 4.4).

Peritoneal macrophages from MMP-2 -/- mice have reduced integrin beta 3 expression

We measured integrin beta3 expression in mouse peritoneal macrophages with RT-PCR and immunofluorescence staining. These techniques demonstrated that the mRNA level of integrin beta 3 was reduced by 58% in peritoneal macrophages of MMP-2-/- mice (P < 0.05; Figure 4.5). Immunofluorescence staining and flow cytometry demonstrated a significant reduction of integrin beta 3 expression in MMP-2 deficient peritoneal

macrophages as well ($P < 0.05$; Figure 4.6; 4.7). These data suggest that the reduced adhesion, which was observed in MMP-2 deficient macrophages, might be due to the reduced expression of integrin beta 3, but not be due to the absence of interaction between these two proteins.

IV. Discussion

The recruitment of macrophages is a common and prominent characteristic of both AAA and atherosclerotic tissue. In these diseases, the invasion of macrophages and release of cytokines and proteolytic enzymes are critical in the initiation and progression of vascular remodeling. Cell adhesion is the initial step in the trans-mural infiltration of macrophages. Integrins are the pivotal receptors that mediate cell-to-ECM and cell-to-cell adhesion. In particular, integrin alpha v beta 3 facilitates the activation of MMP-2 by localizing pro-MMP-2 on the cell surface.

There is evidence that the expression and activation of integrin alpha v beta 3 can not only correlate with other cell types, such as invasive tumor cells, but also correlate with the activation of MMP-2 and MMP-14.[26,184] The binding ability of MMP-2 to integrin alpha v beta 3 is independent of the pathway that activates MMP-2. A disruption of this mechanism has been shown to inhibit angiogenesis and tumor growth, however, the details of the mechanism has not been elucidated.[107,184] Another study suggests that the disruption of MMP-2 binding to integrin alpha v beta 3 inhibits the invasion of mouse peritoneal macrophages.[214] In addition to data concerning the outcome in the inhibition of MMP-2/integrin v beta 3 binding, a recent study demonstrated that the beta3 antagonist m7E3 (abciximab) reduced the activity of MMP-2 and MMP-9 as well as smooth muscle cell migration in rats.[10]

A currently accepted model of integrin mediated cell migration is that a ligand or signaling induced by another agonist might bind to the cytoplasmic domain of the alpha-subunit and induce a conformational change by splitting the cytoplasmic domains of the alpha and beta subunits. In the inactive form, the

alpha-subunit inhibits the interaction between the beta-subunit and the cytoskeletal components. Consequently, this inhibition can be removed by ligand binding. The rate of binding between integrins and ligands is increased during integrin clustering. Intrinsically, the binding to ligands with multiple binding sites or by cytoskeletal interactions may lead to the clustering of integrins. [88,207] Subsequently, the integrins associate with alpha-actinin, leading to a stable interaction with the cytoskeleton.[171]

As mentioned previously, integrins are in a constitutively active conformation in adherent cells. Our findings indicate that MMP-2, as a ligand of integrin alpha v beta 3, might influence macrophage behaviors indirectly through the expression of integrin beta3 or directly by interacting with integrin alpha v beta 3. Chapter 1 discussed the importance of many studies that demonstrated MMP-2 facilitated cell migration through a cleaving the ECM. However, there are controversial opinions about the role of MMP-2 in cell adhesion.[57,167,217] This discrepancy may be due to the various ECM and cell types used, but most likely is linked to the limited understanding of the processes and mechanisms influencing adhesion turnover in migrating cells. It was reported that MMP-2 influences neutrophil-endothelial adhesion by cleaving the latent endothelin-1 (ET-1) to the potent ET-1 in vitro.[58,59] Mouse macrophages express MMP-2 in a relative small amount as compared to MMP-9 (Figure 3.5). However, in the present study, we demonstrated for the first time that MMP-2 deficiency reduced adhesion activity of mouse peritoneal macrophages. This reduction in adhesion might be due to a reduction in integrin beta 3 expression.

Future studies are required to determine the influence of MMP-2 on other adhesion molecules, such as selectins, VCAM, ICAM, and other integrins. Also, more experiments are required to determine the role of MMP-2 in the regulation of other cell behaviors such as survival and apoptosis as well.

Although the exact mechanism underlying the reduced integrin beta 3 expression on MMP-2 deficient macrophages is unknown, several hypotheses may account for this observation. First, MMP-2 might upregulate integrin expression by influencing the activity of certain growth factors. As mentioned

previously (in Chapter 1), MMP-2 can modify the activity of many growth factors through multiple pathways. For example, MMP-2 facilitates the release of TGF-beta by degrading the collagen-associated proteoglycan decorin, which is an anchor protein of TGF- beta. MMP-2 can proteolytically cleave and activate latent TGF-beta as well. Since TGF-beta is able to upregulate the expression of integrins,[220] MMP-2 might upregulate integrin expression via increasing TGF-beta activity. Moreover, MMP-2 may decrease the activity of basic fibroblast growth factor (bFGF) by cleaving the FGF receptor-1. There are controversial opinions for the role of bFGF in the expression of integrin alpha v beta 3 in microvascular endothelial cells. Klein et al. reported that bFGF decreased alpha v beta 3 expression. [109] On the contrary, Sepp et al. demonstrated that bFGF increased alpha v beta 3 integrin expression. [179] It is reported that recombinant VEGF is able to upregulate integrin alpha v beta 3 expression in human microvascular endothelial cells.[178] However, there is not evidence that VEGF activity can be influenced by MMP-2. Taken together, the reduced integrin beta 3 expression on MMP-2 deficiency macrophage might be due to the loss of local MMP-2 control on growth factors, especially, TGF-beta and bFGF. Another potential mechanism is based on the evidence that the expression of integrins can be controlled by the interactions between cells and their surrounding ECM.[44] The degradation of the ECM by MMP-2 might influence the integrin expression of cells. Other leukocytes, especially T lymphocytes, play important roles in vascular diseases. In this study, we did not determine the influence of MMP-2 on other types of inflammatory cells. Future studies are required to test these hypotheses.

We detected a tendency of enhanced production of MMP-9 in mouse peritoneal macrophages of MMP-2 deficient mice. This discovery is supported by recent evidence showing a 10-fold increase in the inactive form of MMP-9 in macrophages by gene transfection did not reduce the rupture of atherosclerotic plaques in carotid arteries of mice (Gough P. presented in the 6th annual conference on Atherosclerosis, Thrombosis and Vascular Biology, 2005). Although MMP-9 can be activated by several serine proteinases, it was reported

that proMMP-9 activation was accomplished by the active forms of MMP-2. These forms are generated after the activation of TIMP-2-free proMMP-2 on the surface of cancer cells. Regarding that study, we concluded that MMP-2 might be important in the regulation of macrophage behaviors by interacting with integrin alpha v beta 3. Therefore, MMP-2 is involved in cell adhesion and invasion through multiple pathways, not just its ability to digest the ECM.

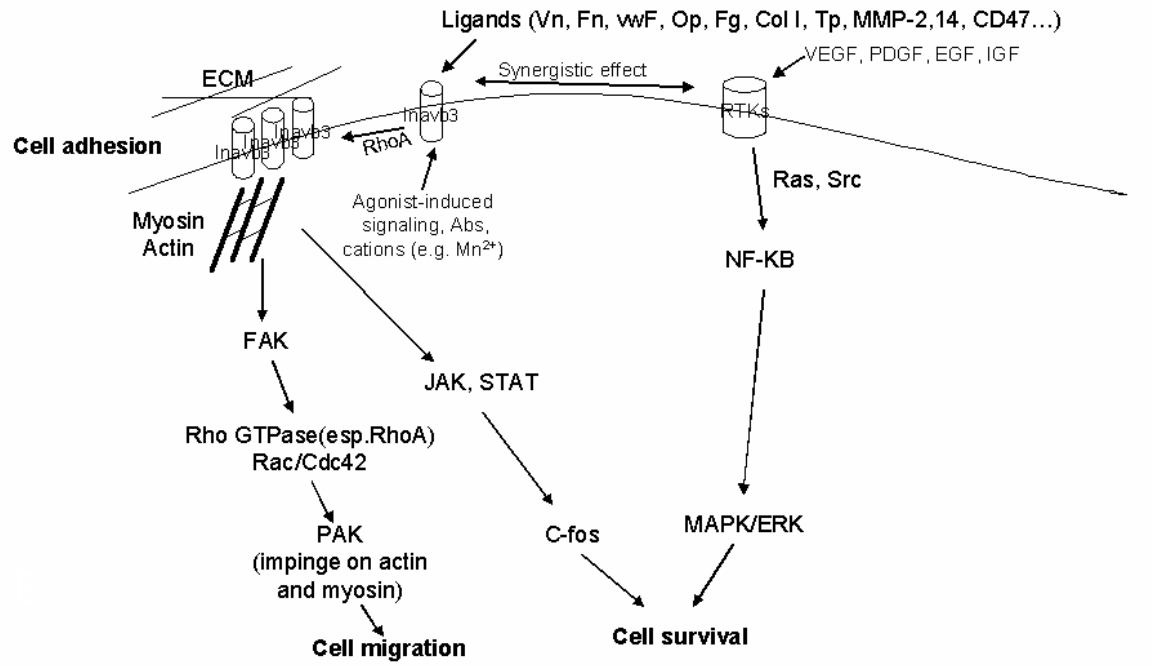


Figure 4.1 Pathways outlining the influence of integrin alpha v beta 3 on cell behaviors. Integrins mediate cell adhesion to ECM and to other cells through activating a variety of protein tyrosine kinases like focal adhesion kinase (FAK) and Src-family kinases. Integrins also initiate intracellular signaling molecules that control cell shape, adhesion, migration, proliferation, differentiation and survival. In adherent cells, integrins are apparently in a constitutively active confirmation. Interaction between integrins and receptor kinases (RTKs) affects cell migration and surface expression levels of integrins.

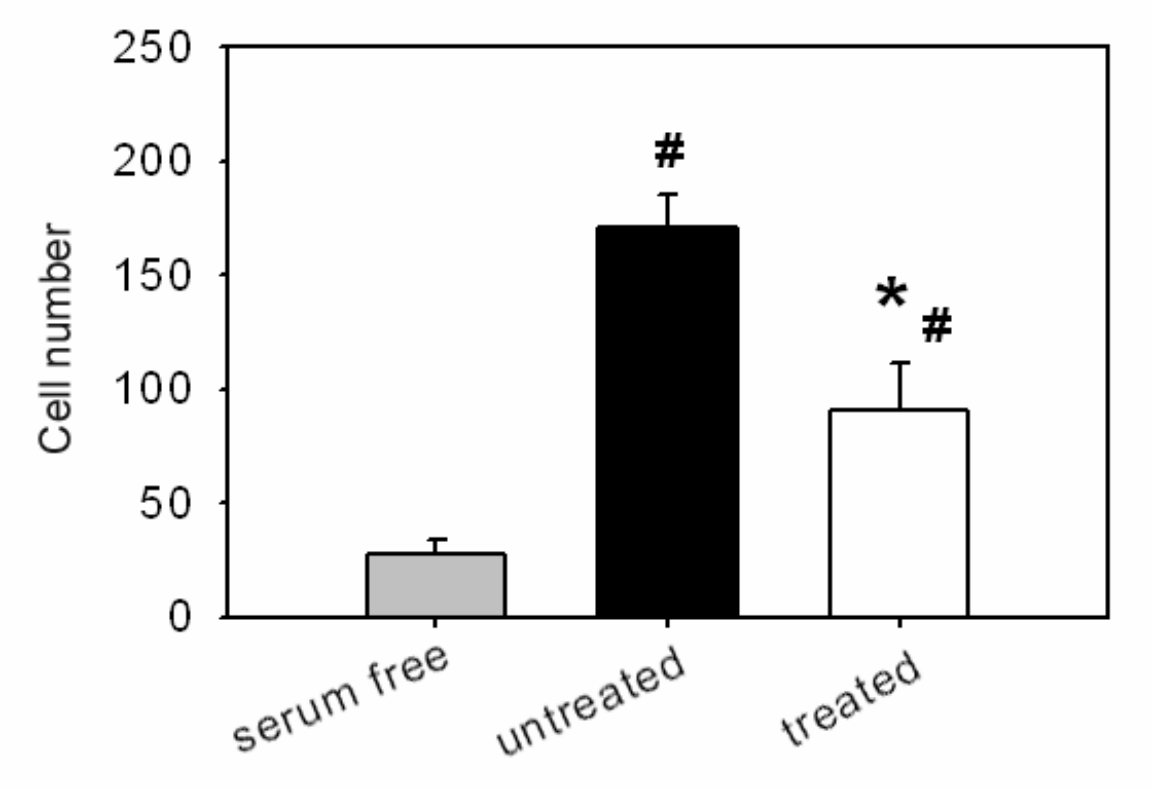


Figure 4.2 The influence of MMP-2 and integrin alpha v beta 3 interaction on macrophage invasion Numbers of macrophages invading through matrigel in the presence of no serum,10% FBS, or 10% FBS with TSRI265 (n = 4 wells per group). Histobars represent the means and bars are SEM. *P < 0.01 for comparisons of TSRI265 treated versus untreated MPMs; $^{\#}P$ < 0.01 for comparisons of wells with FBS versus wells without FBS

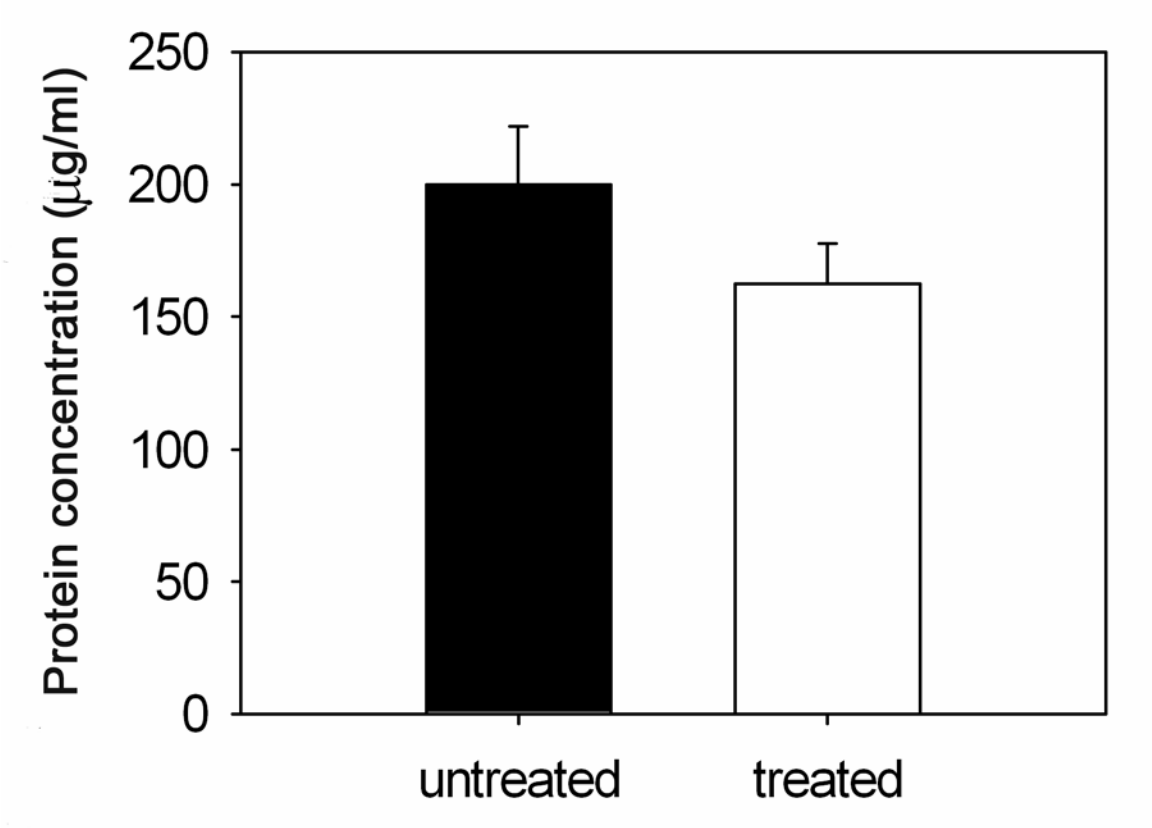


Figure 4.3 Disruption of MMP-2 binding to integrin alpha v beta 3 did not influence macrophage adhesion. BCA protein assay for macrophages shows attachment to the plate after 3 hours incubation either with or without TSRI265 10μM and washing (n = 6 wells per group) Histobars represent the means and bars are SEM.

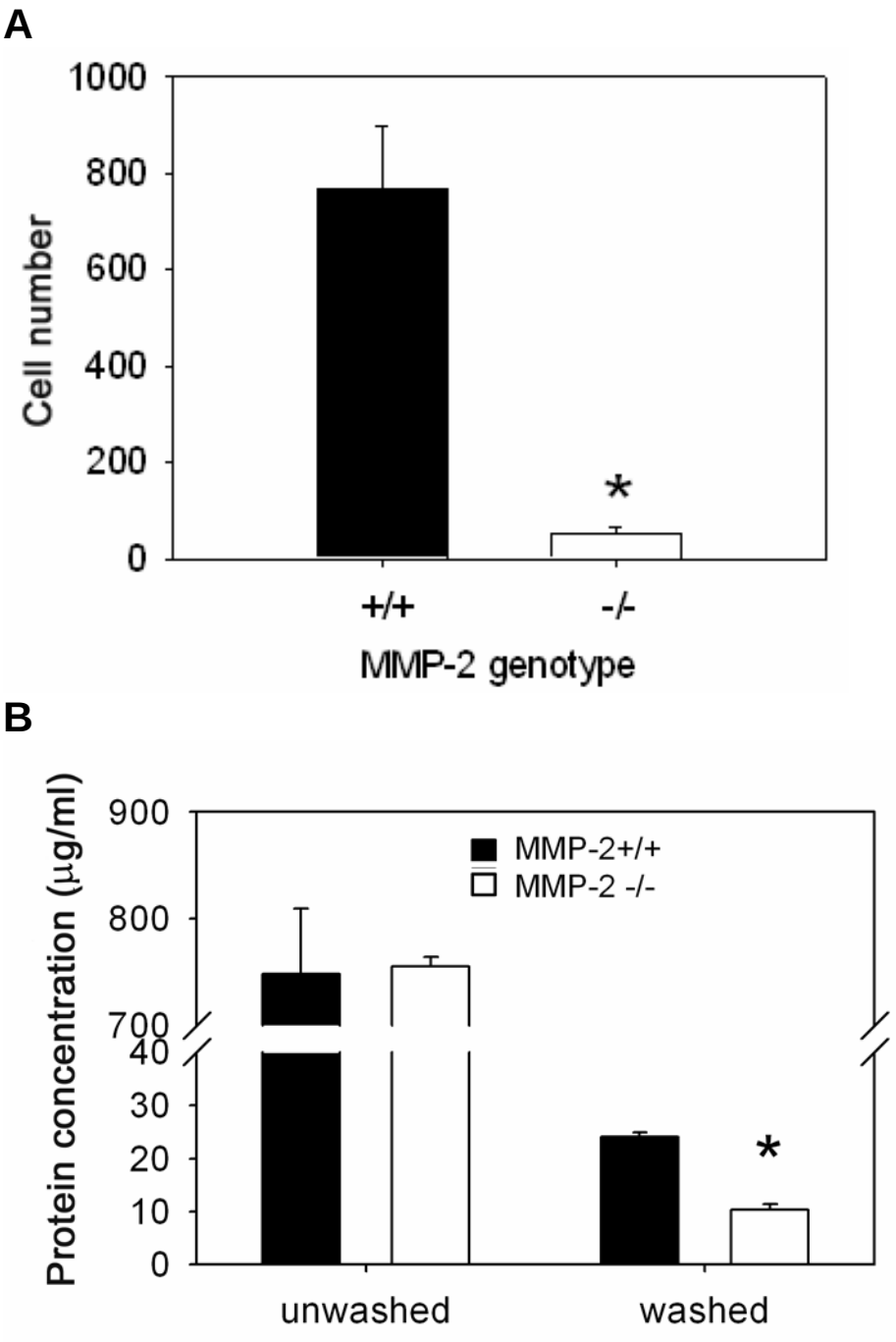


Figure 4.4 Reduced adhesion activity of MMP-2 deficient macrophages (A) After 30 minutes culturing and washing, the macrophages attached in the plate were counted. (n = 5 wells per group) (B) BCA protein assay for the macrophages attached in the plate after 30 minutes incubation and washing (n = 3-5 wells per group; *P < 0.001 for comparisons of MMP-2 wild type versus MMP-2 deficient macrophages) Histobars represent the means and bars are SEM.

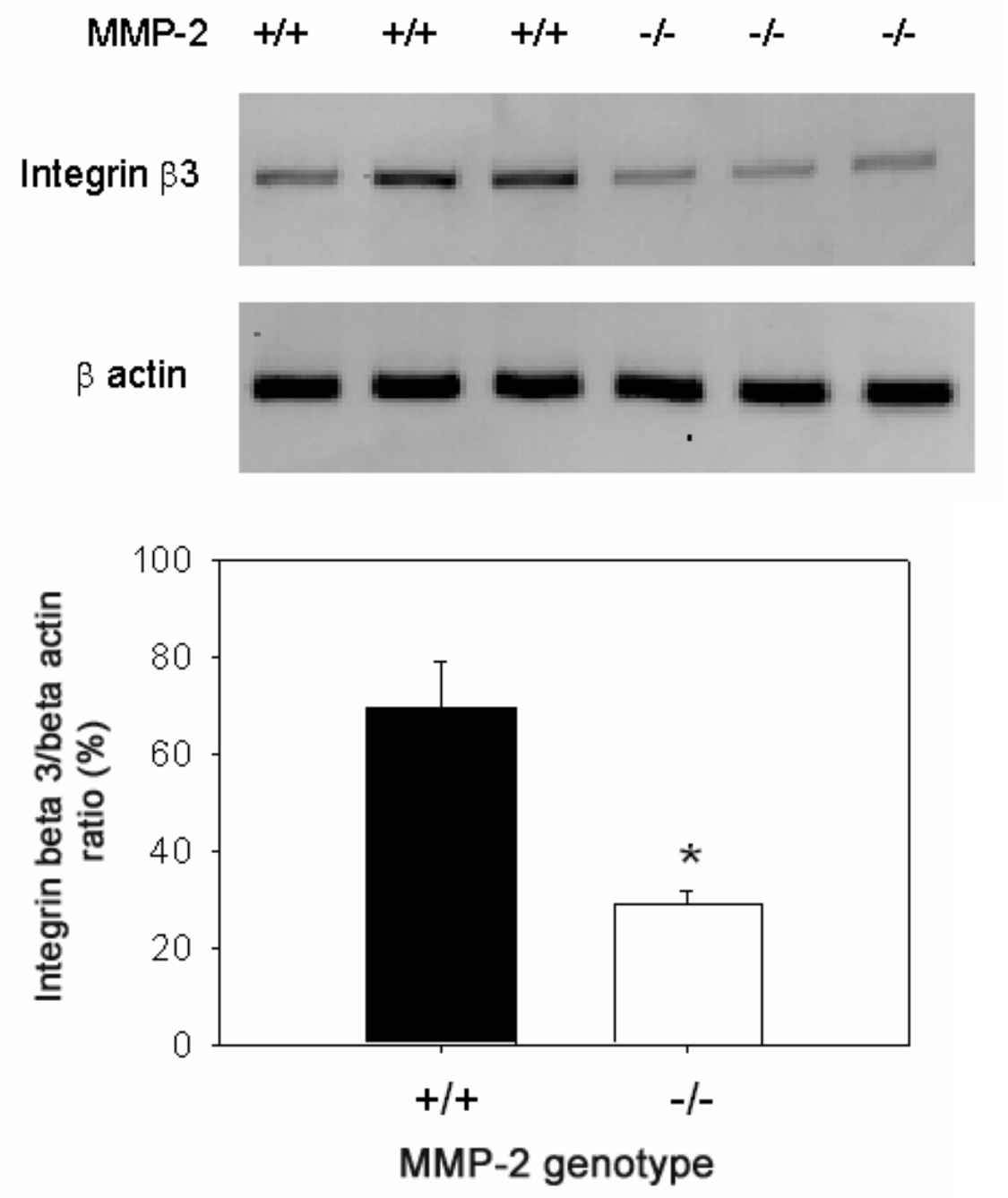


Figure 4.5 RT-PCR of integrin beta 3 expression in MMP-2 deficient macrophages. RT-PCR was performed to semi-quantify the integrin beta3 mRNA from macrophages of MMP-2 wild type and deficient mice. The expected amplicons for integrin beta3 were less pronounced in MMP-2 deficient macrophages compared with MMP-2 wild type macrophages. Beta actin mRNA signals were used as loading controls.

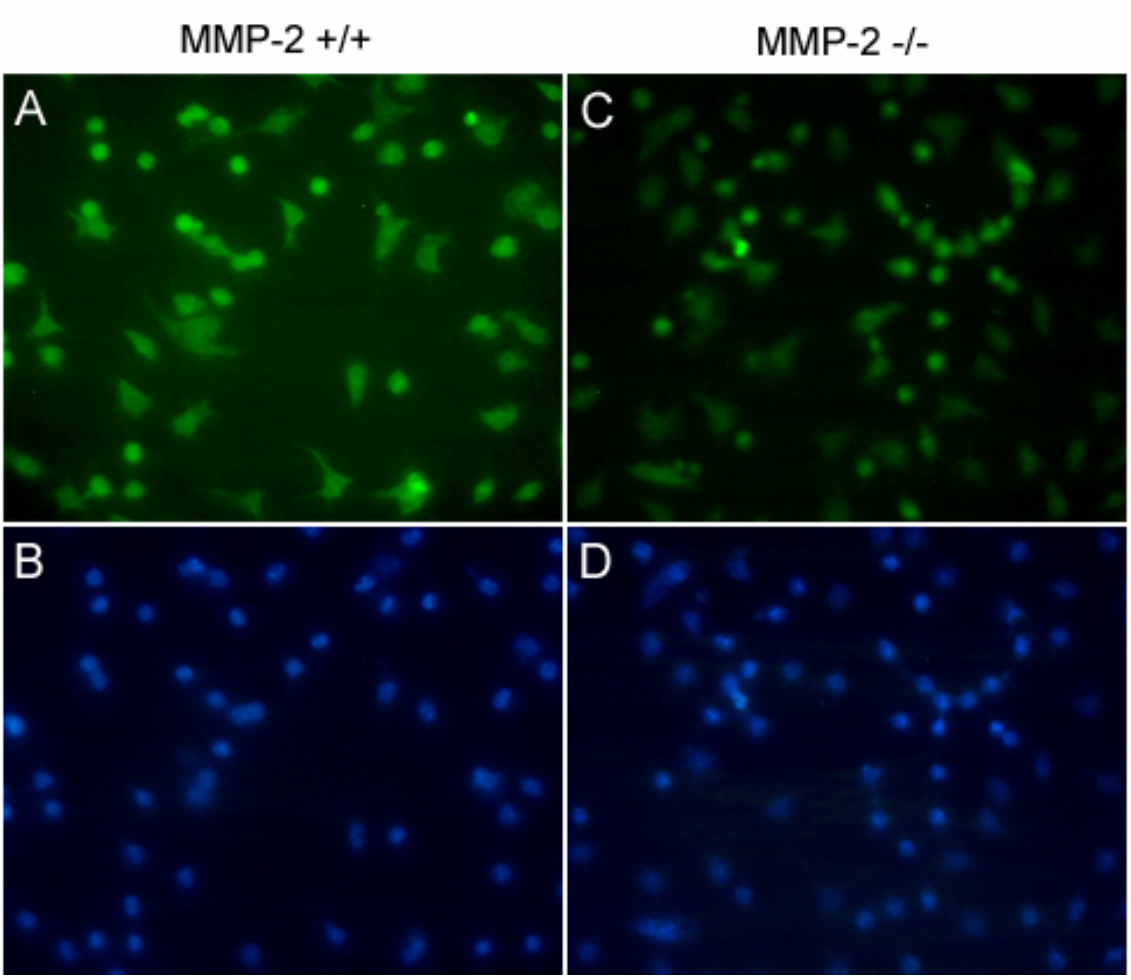


Figure 4.6 Immunofluorescence staining of integrin beta 3 expression in MMP-2 deficient macrophages. Immunofluorescence staining for integrin beta 3 on macrophages was performed. Staining was much less pronounced in macrophages of MMP-2 deficient mice (B) as compared with macrophages of MMP-2 wild type mice (A). Hoechst nuclear staining was performed in the same field of A (C) and B (D), respectively.

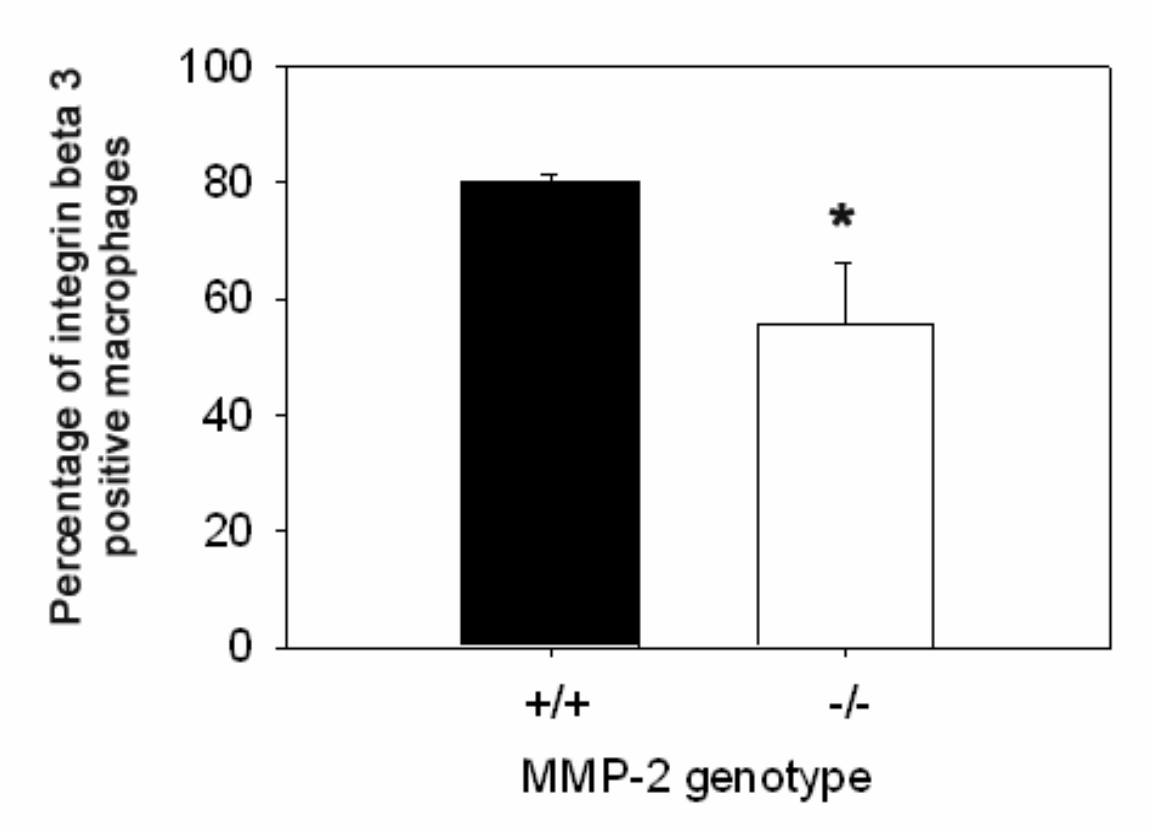


Figure 4.7 Flow cytometry of integrin beta 3 expression in MMP-2 deficient macrophages. The percentage of integrin beta 3 expression positive peritoneal macrophages was reduced in MMP-2-/- mice compared to wild type mice (n = 4/group; $P < 0.05$).

Chapter Five

The Effects of MMP-2 Deficiency in Bone Marrow-Derived Cells on AngII-Induced AAAs and Atherosclerosis in ApoE-/- Mice (*in vivo*)

I. Background

There are two common features in the pathogenesis and evolution of abdominal aortic aneurysms (AAAs) and atherosclerosis: infiltration of inflammatory cells (especially macrophages and lymphocytes) into the vascular wall and remodeling of the extracellular matrix (ECM) at the site of inflammation.[63,170] These two pathologic processes collaborate to form a vicious cycle in the progression of vascular diseases. Proteases and cytokines are enhanced by infiltrative macrophages, and this elaboration accelerates ECM degeneration; vice versa, the degradation of ECM components facilitates the infiltration of inflammatory cells into the vascular wall and leads to an elaboration of proteases and cytokines in situ.[49,157] Therefore, proteases and cytokines might serve the primary linkage of this cycle.

As a protease, MMP-2 (gelatinase A) is involved in cell-to-cell and cell-to-matrix interactions in many physiological and pathological processes such as wound healing, cancer metastasis, and inflammatory joint diseases. MMP-2 first attracted attention in cardiovascular diseases primarily because of its ability to digest a diverse array of ECM components (including elastin and collagen) of the vascular wall. Furthermore, it was reported that MMP-2 facilitated neutrophil-endothelial cell adhesion through cleaving pro-endothelin into an active form in vitro.[58] Our previous study (in Chapter 4) demonstrated that macrophage-derived MMP-2 regulates macrophage adhesion and invasion through interacting with integrin alpha v beta 3. All of the above suggests that MMP-2 is not only involved in the remodeling of the ECM via the proteolysis of ECM components, but also

may be implicated in the infiltration of inflammatory cells into the vessel wall by modulating cell behaviors.

The bone marrow transplantation technique provided a useful approach to examine the contribution of hematopoietic cell gene expression to diseases. Bone marrow transplantation in the apoE-/- and LDLr-/- mouse models has been well established as a method for examining the effect of protein expression by inflammatory cells on the development of cardiovascular diseases. The repopulation of peripheral blood leukocytes by the transplanted bone marrow-derived cells can be confirmed. [14,124] It was also reported that the lethally irradiated mice without bone marrow transplantation died within 14 days after the irradiation.[162]

In this study, we addressed the hypothesis that the MMP-2 produced by bone marrow-derived cells might play an important role in AngII-induced vascular diseases *in vivo*. MMP-2 gene targeted mice were used to achieve this purpose as bone marrow donors.

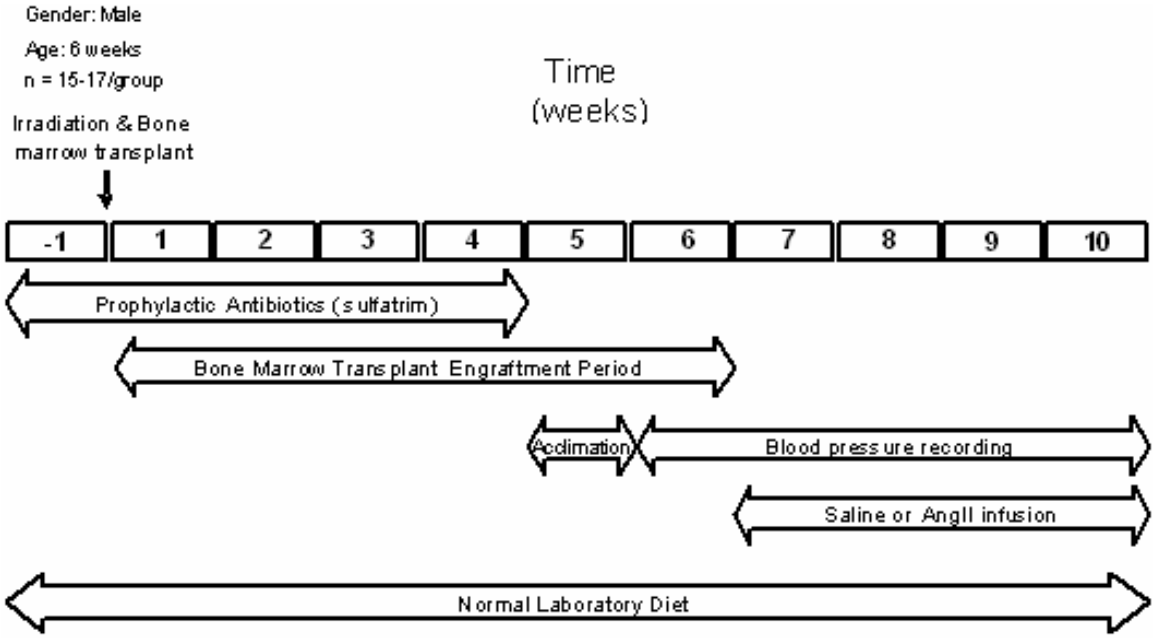
II. Materials and Methods

Animals

MMP-2-/- mice and apoE-/- mice were mated and bred. F1 (MMP-2+/- x apoE+/-) mice were mated to produce littermates of MMP-2+/+ x apoE-/- and MMP-2-/- x apoE-/- mice (F2). Six- week-old male MMP-2+/+ and MMP-2-/- x apoE-/- littermates were used as bone marrow donors. These mice were genotyped by PCR as described previously (in Chapter 2). Six-week-old male apoE-/- mice were used as bone marrow-derived cell recipients.

Experimental protocol

The experiment was designed as follows:



Bone marrow-derived cell transplantation

One week prior to irradiation and bone marrow transplantation, mice were given prophylactic treatment with the antibiotic sulfatrim (4 mg/ml) via their drinking water. ApoE-/- recipient mice were irradiated with 900 rads from a cesium γ source, given as a single dose. Bone marrow was harvested from the femurs and tibias of MMP-2-/- and their littermate MMP-2+/+ x apoE-/- (n = 4/group) mice by flushing with Hank's buffered saline solution (1 ml). Cells were washed, re-suspended, and counted. Irradiated recipient apoE-/- mice (n = 14-17/group) were injected intravenously via the tail vein with 7 x 10^6 bone marrow cells from donor mice. The mice were maintained on antibiotic water for four weeks following irradiation. After an interval of 6 weeks to permit hematopoietic engraftment, the study was initiated.

All of the following experiments were performed as described in Chapter 2:

PCR (polymerase chain reaction) for mouse genotyping

AngII infusion and blood pressure measurement

Peripheral white blood cell counting

Serum lipids and lipoprotein determination

Quantification of atherosclerosis

Classification of AAAs

Statistics

Data are presented as means ± SEM. Two-way ANOVA (two factor analysis of variance) was performed to analyze experiments with two independent variables (i.e. AngII treatment and genotype of MMP-2). One-way ANOVA was performed to test the equality of three or more means with one independent variable at one time by using variances. Student's t-test was used to test the difference between two groups. All of the statistical analyses as above were done by a SigmaStat 2.03 software (SPSS Inc.). A repeating ANOVA was performed to analyze the data of the systolic blood pressure of the mice using a SAS software. Fisher exact probability test was used to determine differences between groups in the incidence of aneurysm formation. Values with $P < 0.05$ were considered statistically significant.

III. Results

Repopulation of irradiated apoE-/- mice with MMP-2+/+ or MMP-2-/- bone marrow-derived cells

Gelatin zymography of aortic extracts was performed to confirm genotypes of the donor mice. As predicted, the band of gelatin lysis by MMP-2 was not visualized in the extracts of aortas of MMP-2 deficient mice. The bone marrow of the recipient mice was harvested at the time of sacrifice and genotyped by PCR. The 1.1 kb amplified DNA product of mutant allele was detected only in bone marrow derived cell DNA from MMP-2-/- bone marrow recipient mice, but not in the DNA from MMP-2+/+ bone marrow transplanted control mice. This suggests that the transplanted bone marrow cells successfully repopulated the bone

marrow of recipient mice (Figure 5.1).

Effect of bone marrow transplantation and AngII infusion on blood composition of the recipient mice

Blood was collected via retro-orbital bleeding and analyzed (n = 9 -11 mice were chosen randomly for each group) one day before the termination of the experiment (Table 5.1). MMP-2 genotypes of the bone marrow-derived cells did not lead to a significant difference in the number of white blood cell (WBC), red blood cell (RBC), hemoglobin (Hb) or platelet. AngII infusion did not change peripheral blood WBC and platelet count. Interestingly, AngII infusion caused a significant rise in the number of RBC and a corresponding increase in Hb level for both MMP-2 deficient and MMP-2 wild type bone marrow cell recipient mice ($P < 0.001$).

Effect of bone marrow transplantation and AngII infusion on the body weight of the recipient mice

The recipient mice did not gain body weight as normal mice due to the irradiation (Figure 5.2; n = 14 -16/group). There was a significant loss of body weight in AngII infused mice compared with saline infused mice (-2.2 ± 0.3 g vs. 1.3 ± 0.3 g; $P < 0.001$). However, there was no statistical difference between MMP-2+/+ and -/- bone marrow-derived cell recipient mice ($P = 0.499$).

Effect of bone marrow transplantation and AngII infusion on the blood pressure of the recipient mice

Systolic blood pressure was measured in conscious mice using a computerized non-invasive tail-cuff system, 5 days per week (n = 10 -11/group). Saline infusion did not change systolic blood pressure of the recipient mice. AngII infusion increased systolic blood pressure in both MMP-2+/+ and -/- bone

marrow-derived cell recipient mice ($P < 0.0001$). There was a significant change of blood pressure caused by the interaction between AngII infusion and time within the AngII treated mice ($P < 0.001$; Figure 5.3). Neither MMP-2 genotype in bone marrow cells caused significant differences in blood pressure.

Effect of bone marrow transplantation and AngII infusion on the cholesterol profile of the recipient mice

The genotype of MMP-2 of bone marrow-derived cells and AngII infusion did not cause statistically significant difference in total serum cholesterol concentrations (TC). For MMP-2+/+ bone marrow recipients, the TC was 183 ± 12 mg/dl in saline group vs. 204 ± 20 mg/dl in AngII group; for MMP-2-/- bone marrow recipients, the TC was 161 ± 6 mg/dl in saline group vs. 190 ± 21 mg/dl in AngII group. There was a mild increase of TC in AngII infused mice (n = 9 - 10/group; P = 0.138; Figure 5.4). No significant difference was detected in lipoprotein distribution in MMP-2+/+ and MMP-2-/- bone marrow transplanted mice (Figure 5.5).

Effect of bone marrow transplantation on AngII-induced hypertrophy of the aortic arch of the recipient mice

AngII infusion induced significant hypertrophy in the aortic arches of recipient mice. The aortic arch area was increased significantly in AngII-infused mice as compared to saline-infused mice ($P < 0.001$). However, MMP-2 deficiency in bone marrow derived cells did not ablate the hypertrophy (Figure 5.6; n = 10-11/group; P = 0.728). For saline infused mice, the area of aortic arch was 16.0 ± 0.7 mm^2 in MMP-2+/+ bone marrow-derived cell recipients vs. 16.0 ± 0.5 mm^2 in MMP-2-/- bone marrow-derived cell recipients. For AngII treated mice, the area of aortic arch was 21.7 ± 0.8 mm^2 in MMP-2+/+ bone marrow cell recipients vs. 21.2 ± 0.7 mm^2 in MMP-2-/- bone marrow cell recipients.

The repopulation of MMP-2-/- bone marrow-derived cells significantly decreased the atherosclerosis formation in the recipient mice

The extent of atherosclerosis was quantified both by *en face* analysis of the aortic arch and thoracic aorta (Figure 5.7 and 5.8) and sequential cross sectioning of the aortic root (Figure 5.9). Both methods demonstrated a significant reduction of AngII-induced atherosclerosis in MMP-2-/- bone marrow cell-repopulated mice as compared to MMP-2+/+ bone marrow cell-repopulated mice (2.1 ± 0.2% vs. 5.7 ± 0.6% of thoracic aorta and aortic arch area, $P < 0.001$; 7.2 ± 2.5 x 10^{-3} mm^2 vs. 24.5 ± 6.7 x 10^{-3} mm^2 of the lesion area on the transverse sections of aortic root, $P < 0.05$). Saline-infused MMP-2-/- bone marrow transplanted mice demonstrated a tendency toward reduction of atherosclerosis compared to wild type bone marrow recipient mice (0.2 ± 0.0% vs. 0.7 ± 0.2% of thoracic aorta and aortic arch area; 2.9 ± 1.0 x 10^{-3} mm^2 vs. 8.2 ± 2.5 x 10^{-3} mm^2 of plaque area on the transverse sections of aortic root). However, this was not statistically significant.

The repopulation of MMP-2-/- bone marrow-derived cells prevented the AngII-induced AAA formation in the recipient mice

Abdominal aortic aneurysms were not detected in saline-infused recipient mice. AngII infusion (1,000 ng/kg/min) generated AAAs in 53% (nine out of seventeen) of mice with MMP-2+/+ bone marrow derived cells and 12.5% (two out of sixteen) of mice with MMP-2-/- bone marrow derived cells (n = 14 - 17/group; $P < 0.05$; Figure 5.11). There was no statistical difference between saline and AngII infused MMP-2-/- bone marrow derived cell recipients ($P = 0.30$). AAAs were classified into four classes based on the standard that was described in Chapter 2. Both AAAs occurred in MMP-2-/- bone marrow cell recipient mice were type II AAAs. Among MMP-2+/+ bone marrow recipient mice, one type I, five type II, and one type III AAAs developed. In addition, two MMP-2+/+ bone marrow-derived cell recipient mice died from AAA rupture, which is

defined as type IV AAAs, at the 8th and 10th day of AngII infusion, respectively (Figure 5.11). Abdominal aortas were weighed to provide an index of AAA severity. In saline-infused mice, MMP-2 deficiency in bone marrow-derived cells did not influence the weight of the abdominal aorta of mice. However, abdominal aorta weight increased markedly after AngII infusion compared with saline infusion ($P < 0.001$). This increase was significantly attenuated in MMP-2-/- bone marrow-derived cell transplanted mice. The weight of abdominal aorta was 23.2 ± 2.1 mg in MMP-2+/+ bone marrow-derived cell recipients vs. 16.8 ± 1.2 mg in MMP-2-/- bone marrow-derived cell recipients (n = 14 -16/group, $P < 0.05$; Figure 5.12).

IV. Discussion

In this study, our major finding is that MMP-2 deficiency in bone marrow-derived cells decreased the incidence of AngII-induced AAA and the severity of atherosclerosis in apoE-/- mice. Linked with the reduced invasion ability of peritoneal macrophage of MMP-2 deficient mice, this result is rational. The immunohistochemical staining for macrophage indicated a reduction of macrophage content in atherosclerotic plaques of MMP-2-/- bone marrow cell recipient mice. However, this might be due to the reduced plaque size in these mice (Figure 5.10). Further study is required to quantify the reduction and evaluate the significance of the difference.

The apoE-/- mouse model is considered to be the most useful model for atherosclerosis analysis because of its high histological similarity to human diseases. In this model, macrophages are the major cellular component of the atherosclerotic plaque. At the age of 5 to 6-weeks, apoE-/- mice demonstrated monocytic adhesion and trans-endothelial migration to the endothelial surface of the aorta, detected by electron microscopy (EM). At 6 to 10-week-old, most apoE-/- mice have developed fatty-streak lesions comprised primarily of foam cells with a small number of migrating smooth muscle cells. These fatty-streak lesions rapidly progress to advanced lesions, which are typically comprised of a

necrotic core surrounded by proliferating smooth muscle cells and varying amounts of ECM, including collagen and elastin.[145] In AngII-induced AAAs, the infiltration of macrophage into the vascular wall is a predominant feature also found in human disease. Taken together, a reasonable conclusion can be that reduced infiltration of macrophages might influence both atherosclerosis and AAAs development.

However, there was a discrepancy between the results of the current study and those of a previous study, which demonstrated that MMP-2 deficiency was not protective for atherosclerosis and AAAs in apoE-/- mice (in Chapter 2). Several potential mechanisms may explain the contradiction. First, the research community is realizing that results obtained through analysis of gene function in transgenic mice may differ from pharmacological interventions, which are relatively short-termed. The transgenic animal may develop effective adaptations to the long-term change regarding gene expression. For instance, deletion of an isoform of NOS (nitric oxide synthase) led to an up-regulation of other NOS isoforms in mice. In myoglobin null mice, an elevated capillary density was observed which enhances the oxygen supply to tissues.[72] Discrepancies between results from clinical trials using a pharmacological blockade of integrins for cancer therapy and results from animal studies utilizing the genetic deletion of integrins provide an example of the contradiction in methods. This controversy has raised a discussion of the potential role of these integrins in angiogenesis and the safety and efficacy of integrin antagonists. [23] A bone marrow transplantation experiment is similar to a study utilizing short-term pharmacological treatment rather than the transgenic loss of gene function in the animal.

Second, irradiation itself may influence the development of vascular diseases. Ionizing irradiation is a risk factor for the development of atherosclerosis via inducing the release of inflammatory cytokines. Moreover, irradiation injures endothelial cells and increases vascular permeability. In the current study, recipient mice received high dose gamma-irradiation, which might influence the processes of atherosclerosis dramatically. It was reported that total

body gamma irradiation and syngeneic bone marrow (BM) reconstitution increased atherosclerosis area in the aortic root but decreased atherosclerosis area in thoracic aorta in LDLr-/- mice fed with high fat diet for 16 weeks. The reasons for this discrepancy remain unresolved.[176] Unfortunately, there is not a counterpart study in apoE-/- mice. In this dissertation project, we observed that, after AngII-infusion, irradiated and syngeneic BM transplanted apoE-/- mice had a tendency to have larger atherosclerotic plaques in the aortic root when compared to non-irradiated mice ($24.5 \pm 6.7 \times 10^{-3}$ mm^2 vs. $10.0 \pm 3.8 \times 10^{-3}$ mm^2; $P = 0.114$). There was a significant difference in the percentage of atherosclerotic lesion to total surface area of aortic arch between irradiated and BM transplanted mice and non-irradiated mice ($6.7 \pm 0.4\%$ vs. $3.1 \pm 0.8\%$; $P < 0.001$). These unpublished results affirm the effect of irradiation in human atherosclerosis. We do not have saline control in the non-irradiated mice because of limitations in the number of MMP-2 x apoE-/- mice available.

Irradiation might exacerbate inflammatory cell infiltration into the vessel wall during atherosclerosis development, thereby, intensifying the beneficial effect of impaired infiltration ability of MMP-2 deficient inflammatory cells. Flanders et al. reported that mice lacking Smad3, which is a critical downstream signaling molecule of TGF-beta, are protected against cutaneous injury induced by ionizing radiation. [61] TGF-beta is a potent chemotactic factor of monocytes, and induces macrophage to secret cytokines. [208] As mentioned before, TGF-beta can be activated by MMP-2. Although there is lack of an investigation in the role of TGF-beta in irradiation-induced atherosclerotic lesion, a reduced local TGF-beta activation by MMP-2 deficient inflammatory cells might be protective.

There is lack of evidence suggest that ionizing irradiation is a risk factor of AAAs. Some clinical studies suggest that beta-irradiation for the treatment of in-stent restenosis does not increase the aneurysm incidence in patients .[196] However, there are several clinical trials which indicate that carotid artery aneurysms can be induced by radiotherapy for head and neck cancer. Histopathology findings of irradiation-induced aneurysm include carotid artery necrosis and an incomplete and fragmented endothelial lining. [152, 177] The

exacerbation of inflammatory cell infiltration into the vascular wall might contribute to irradiation-induced aneurysm formation as well.

The hematopoietic system contributes many cell types including circulating leukocytes, resident macrophages in tissues and organs. Bone marrow transplantation (BMT) technique using gene-targeted mice as donors and recipients provides a useful approach to examine the contribution of hematopoietic cell gene expression for diseases in vivo.

Some experiments suggest that resident progenitor cells in the adventitia are the source of smooth muscle cells in atherosclerotic lesions, and bone marrow progenitor cells hardly differentiate into resident cells in the vascular wall.[87] However, other studies suggest that hematopoietic stem cells can differentiate into vascular cells, which take part in the pathogenesis of atherosclerosis.[173] This controversy might be due to the limitation of techniques used to distinguish and determine the origin of stem cells in tissue and organs. Although there are controversial opinions, it should be noted that bone marrow-derived cells are progenitors that have high plasticity. Therefore, a bone marrow transplantation experiment can be inherently flawed due to an inability to exclude the contribution of other types of cells, such as endothelial cells [215] and VSMCs.[173] Taken together, although it could not change the central conclusions of this study as described above, irradiation and bone marrow transplantation might influence the development of vascular diseases. This might contribute to the apparent discrepancy between the findings of the current study and those of a previous study (in Chapter 2).

Hemodynamic conditions regulate vascular remodeling, monocyte adhesion and transmural migration in murine AAA models.[181] Although AngII infusion dramatically increased mouse systolic blood pressure, MMP-2 deficiency in bone marrow-derived cells did not ablate AngII-induced hypertension. This indicates that the protective effect caused by the deletion of MMP-2 from bone marrow-derived cells was not due to the control of blood pressure.

Interestingly, AngII infusion caused a significant rise in the number of RBCs and a corresponding rise in the Hb levels in the blood of study mice.

Several clinical observations suggest that AngII is responsible for inappropriately sustaining production of erythropoietin despite hematocrit elevation, and is capable of directly stimulating the erythroid progenitors in bone marrow to produce erythrocytes.[75,169] However, the mechanism is unclear. Another study released an opposing result, stating that under physiological conditions in healthy volunteers, increased concentrations of endogenous AngII is not a major factor of erythropoietin regulation. [64] In addition, MMPs/TIMPs are secreted by normal human bone marrow hematopoietic cells and stromal cells and may play an important role in intercellular cross-talk during hematopoiesis.[132] To fully understand the dramatic effects we have observed in the current study, the role of MMP-2 in the regulation of behavior of stem cells should be considered.

Table 5.1 Blood cell compositions of bone marrow-derived cell recipient mice

MMP-2 genotype of BM	MMP-2 +/+		MMP-2-/-	
Infusion	Saline	AngII	Saline	AngII
WBC ($1x10^3$ cells/mm^3)	10.1 ± 0.7	7.9 ± 0.9	8.1± 0.6	7.5 ± 1.1
RBC ($1x\ 10^6$ cells/mm^3)	9.3 ± 0.2	11.5 ± 0.3*	9.6 ± 0.1	11.0 ± 0.8*
Platelets ($1x10^3$ cells/mm^3)	992 ± 36	918 ± 61	1052 ± 40	976 ± 81
Hb (g/dl)	14.3 ± 0.3	16.7± 0.3*	14.5 ± 0.2	17.0 ± 0.5*

Data represent mean ± SEM for groups of 9-11 mice.
*P<0.001 with respect to saline infused mice of the same MMP-2 genotype

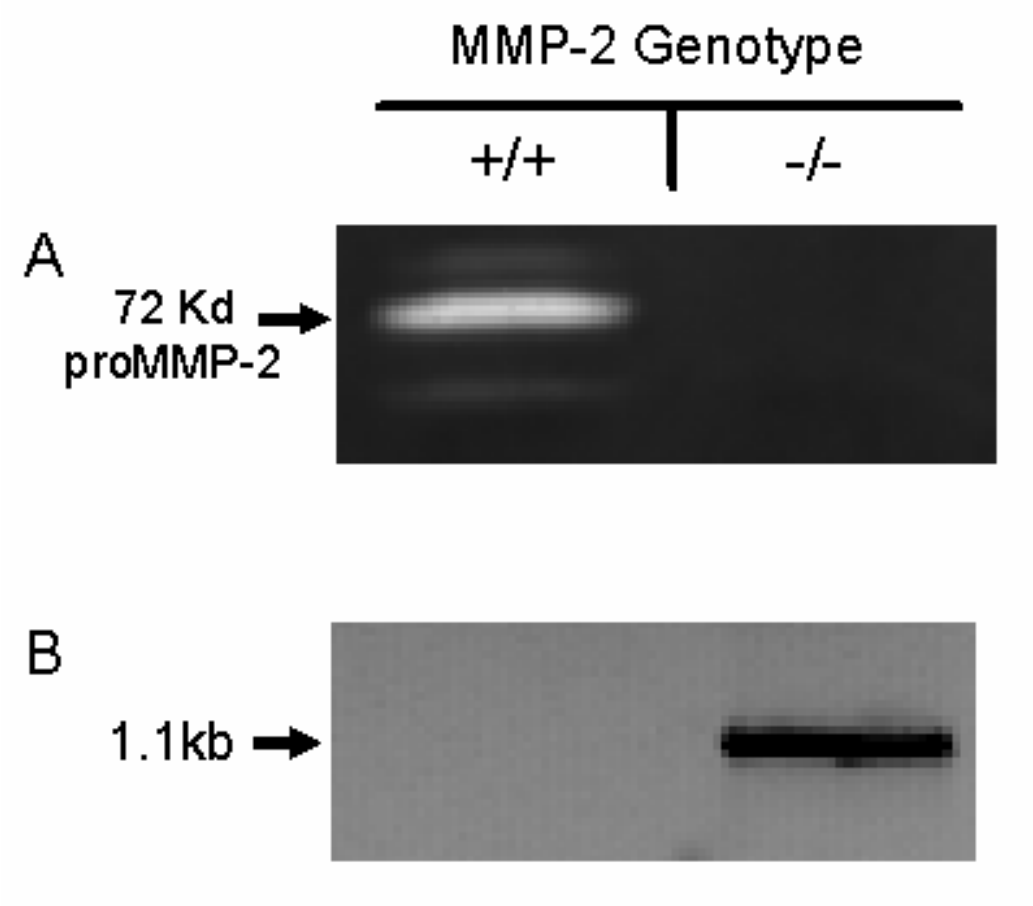


Figure 5.1 Confirmation of MMP-2 genotype of bone marrow donors and recipient mice (A) Gelatin zymography of extracts (15µg) of aortas of MMP-2+/+ and -/- x apoE-/- mice. No MMP-2 expression was detected in MMP-2 deficient mice, whereas a prominent 72kDa band of gelatin lysis by MMP-2 was present in the MMP-2 wild type mice. (B) Representative PCR genotyping of bone marrow removed from recipient mice transplanted with either MMP-2+/+ or -/- bone marrow-derived cells. The 1.1 kbp (for the mutant allele) band was detected in the DNA of bone marrow from MMP-2-/- bone marrow recipient mice and was absent in bone marrow of the control transplanted mice.

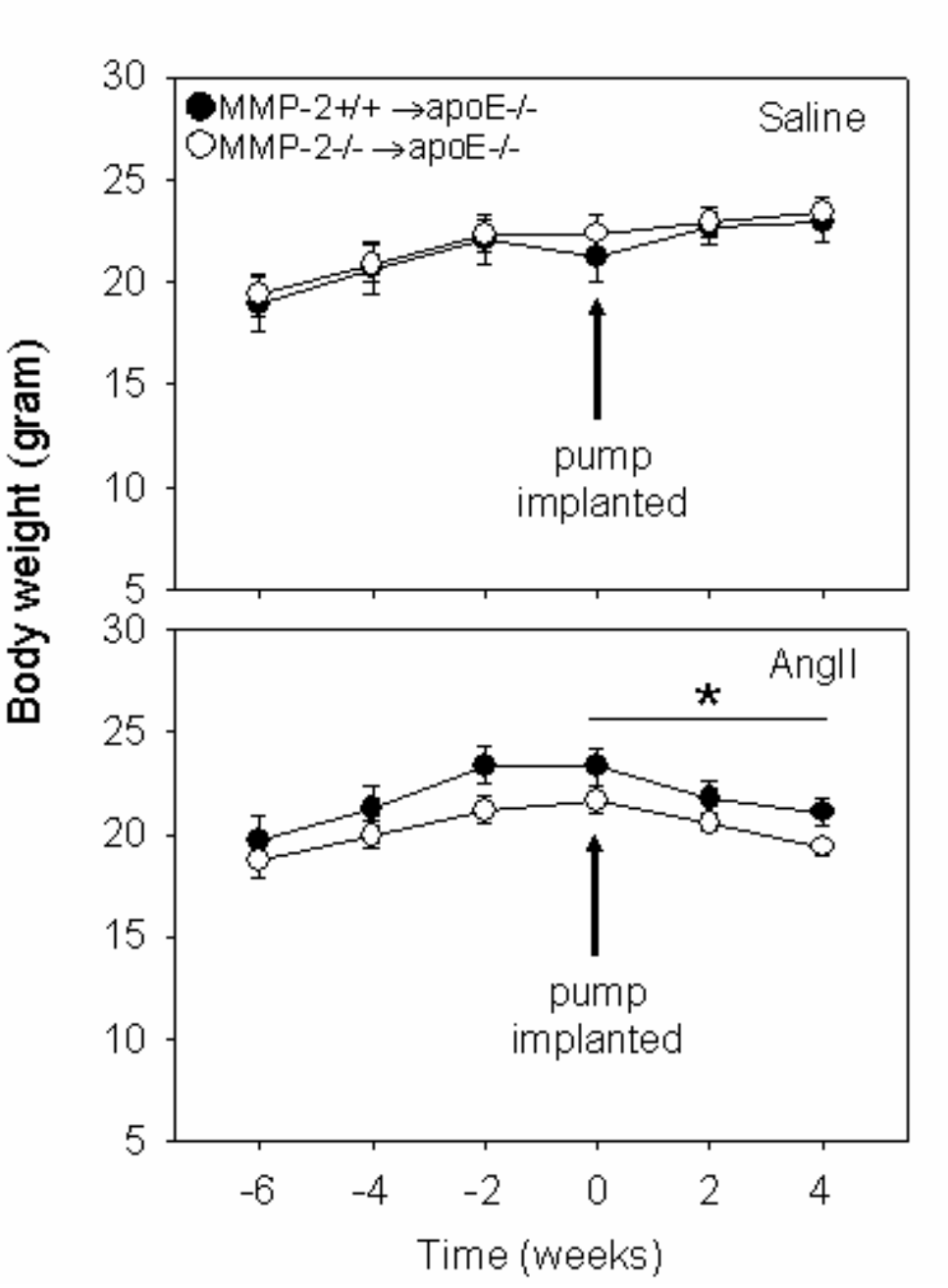


Figure 5.2 Effect of bone marrow-derived cell transplantation and AngII infusion on the body weight of recipient mice. There was no effect of MMP-2 genotype in bone marrow-derived cells on the body weight of the study mice. However, AngII infusion caused a significant loss of body weight compared with saline infusion (*P < 0.001). Points represent the means of 14 -16 mice and bars are SEM.

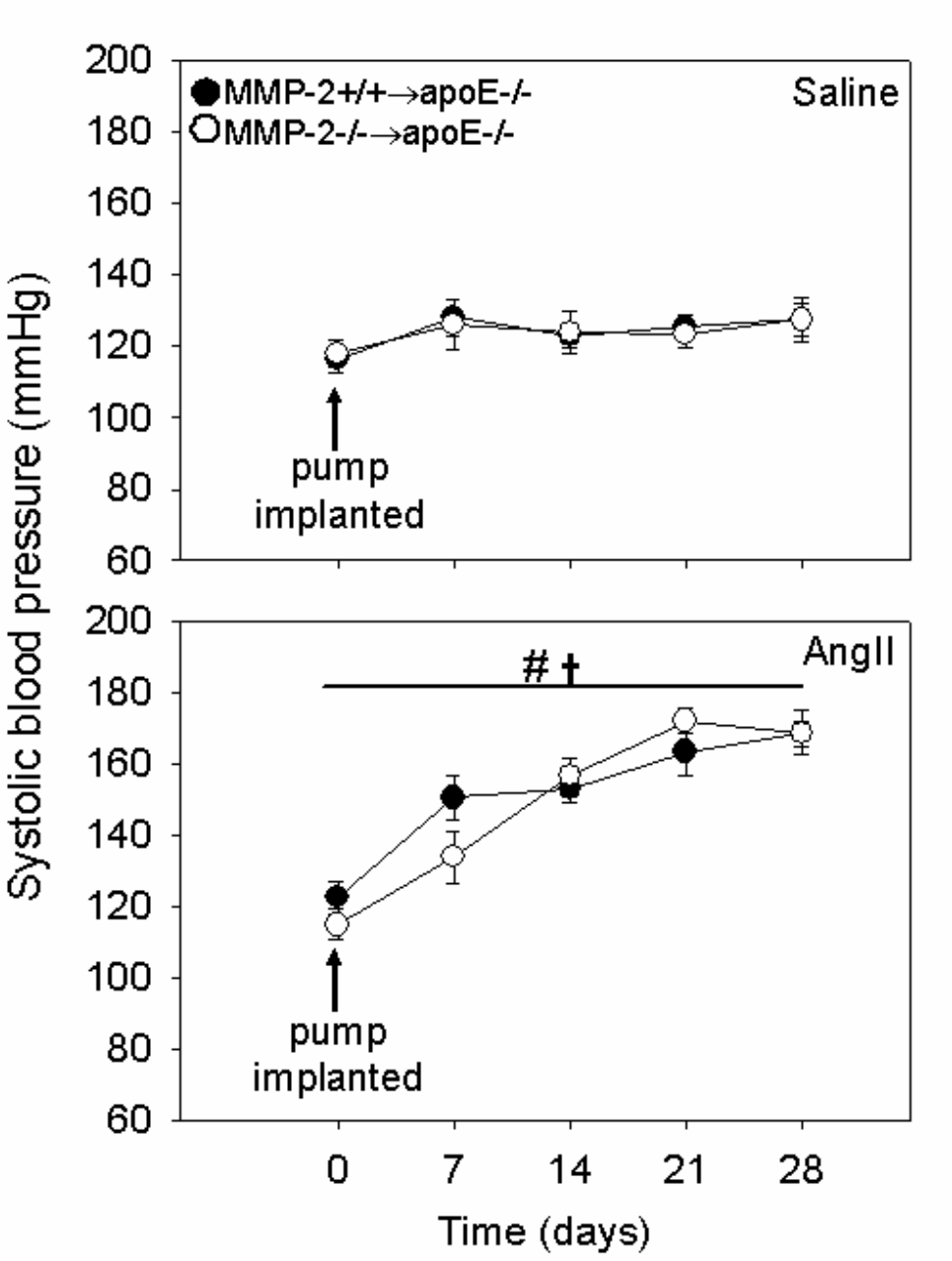


Figure 5.3 Effect of bone marrow-derived cell transplantation and AngII infusion on the blood pressure of recipient mice. Mini-pumps filled with saline or AngII (1,000 ng/kg/min) were implanted subcutaneously into the mice at day 0 (n = 10-11/group). Saline infusion did not change systolic blood pressure of the recipient mice. There was a significant change in blood pressure during AngII infusion in the treated mice. A repeating ANOVA was used to analyze this data (#*P* < 0.0001 for comparisons of AngII versus saline infusion within MMP-2 genotypes; †P < 0.001 for analysis of the interaction between AngII infusion and time within AngII treated mice). Points represent the means of measurements of each week and bars are SEM.

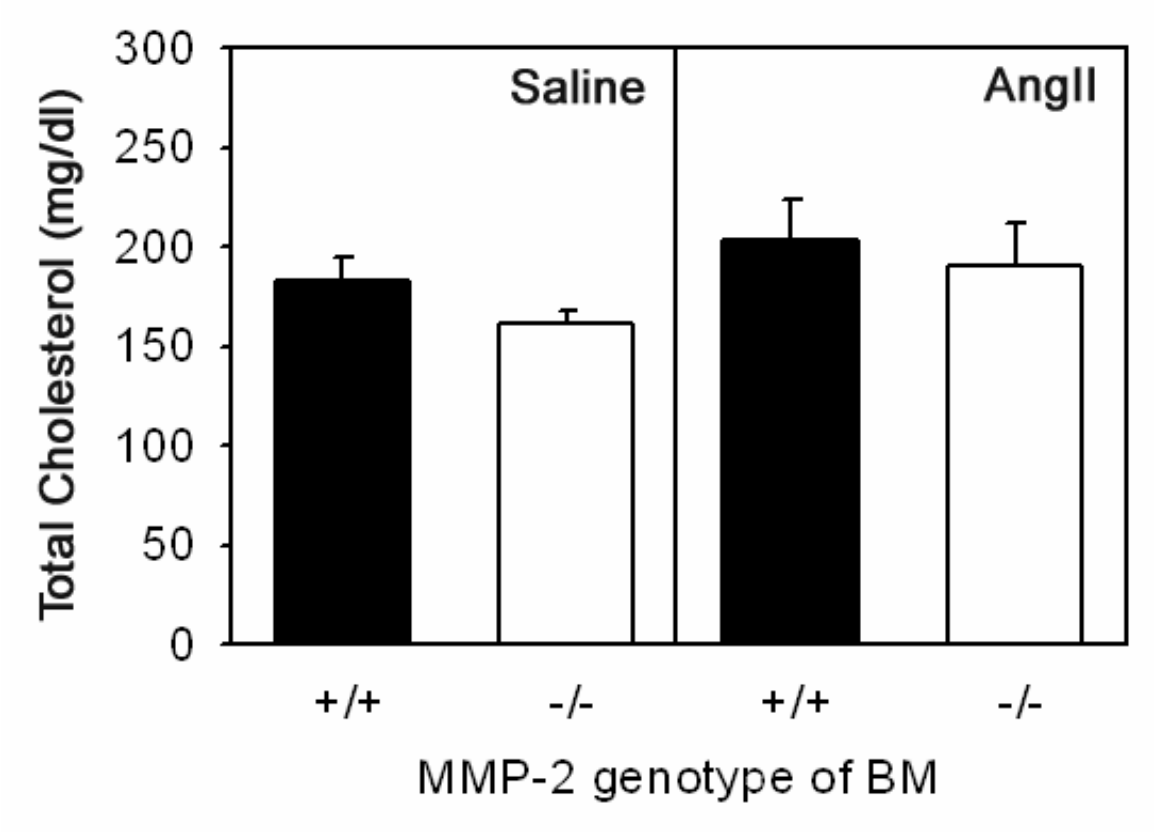


Figure 5.4 Effect of bone marrow-derived cell transplantation and AngII infusion on the total cholesterol plasma concentration of recipient mice. Serum total cholesterol concentrations were measured using commercially available enzymatic assay kits. There was no significant difference in serum cholesterol concentrations across groups (n = 9-10/group). Histobars represent the means and bars are SEM. BM stands for bone marrow.

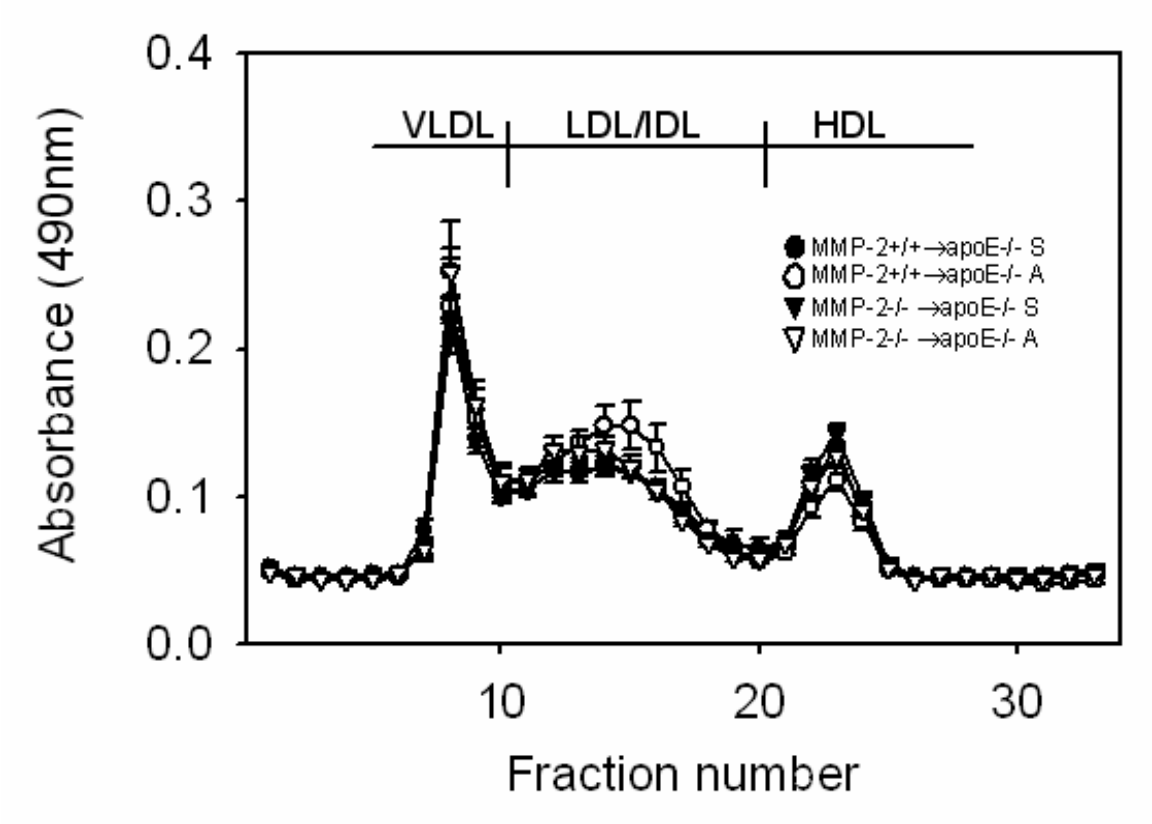


Figure 5.5 Effect of bone marrow-derived cell transplantation and AngII-infusion on the lipoprotein distribution of recipient mice. Lipoprotein distribution of mice was resolved using size exclusion chromatography, namely, fast performed lipid chromatography (FPLC). The curves of VLDL, LDL/IDL and HDL of the mice were overlapped. Points represent the means of 9-10 individual mice, and bars are SEM. S is saline-infused mice; A is AngII-infused mice.

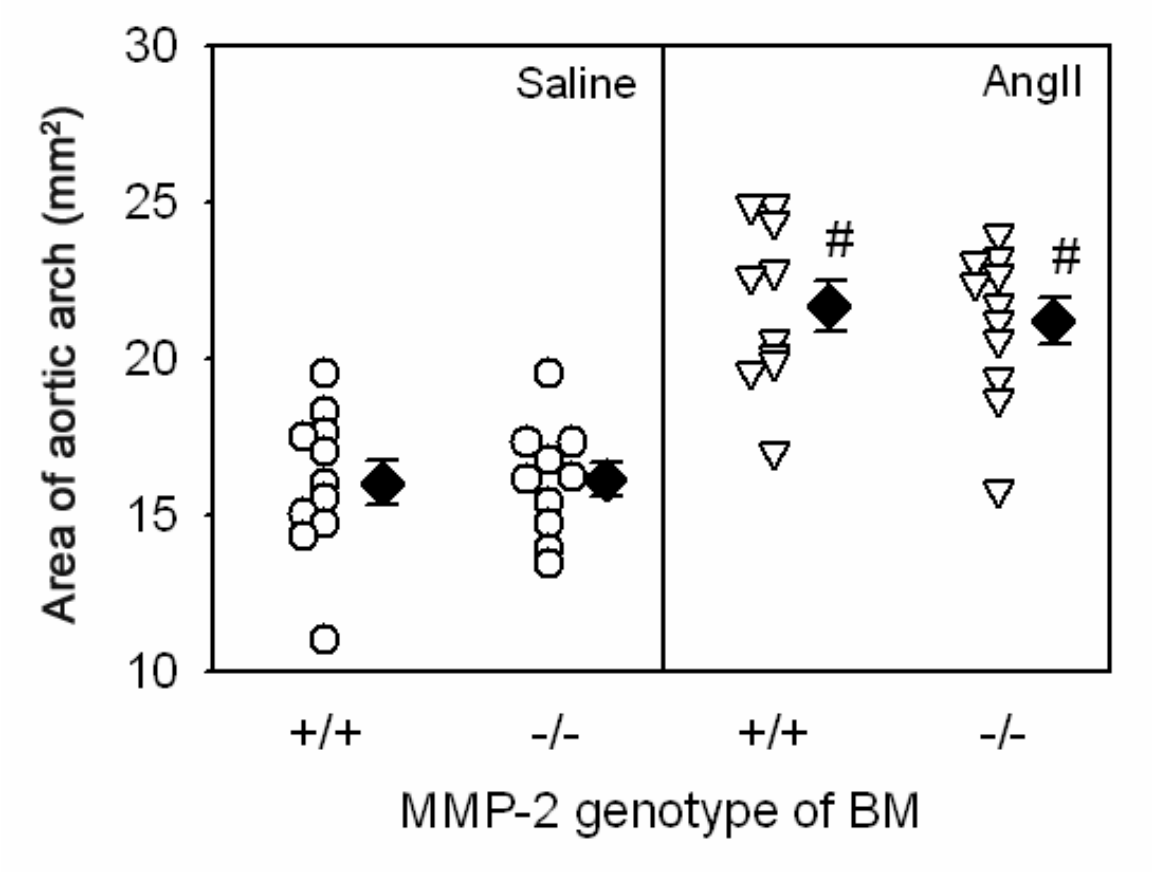


Figure 5.6 Effect of bone marrow-derived cell transplantation on AngII-induced hypertrophy on the aortic arch of recipient mice*.* AngII infusion caused a significant increase in the intima area of the aortic arch of the recipient mice. There was no difference between MMP-2+/+ and -/- bone marrow-derived cell recipient mice. Values obtained from individual mice are represented as open symbols, the means are represented as closed symbols and SEM as bars. (n = 10-11/group; # $P < 0.001$ for comparisons of AngII versus saline infusion within MMP-2 genotypes) BM stands for bone marrow.

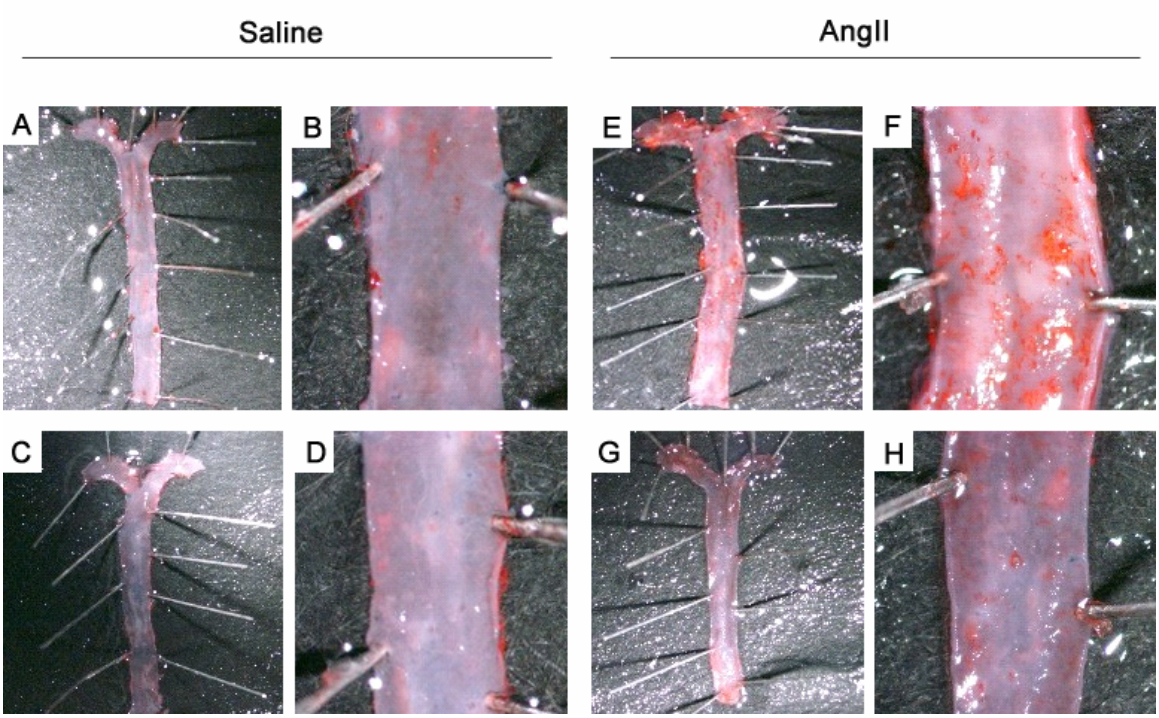


Figure 5.7 Representative light photomicrographs of luminal surface of aortic arch and thoracic aortas of the recipient mice. The aortas were stained with Oil Red O. Red-stained areas are atherosclerotic lesions. AngII-infusion (E and G) accelerates atherosclerosis development in mice as compared to saline infusion (A and C). MMP-2-/- bone marrow cell recipient mice (C and G) have reduced atherosclerotic lesion as compared to MMP-2+/+ bone marrow cell recipient mice (A and E). B, D, F, and H show the detail of boxed area in A, C, E, and G, respectively.

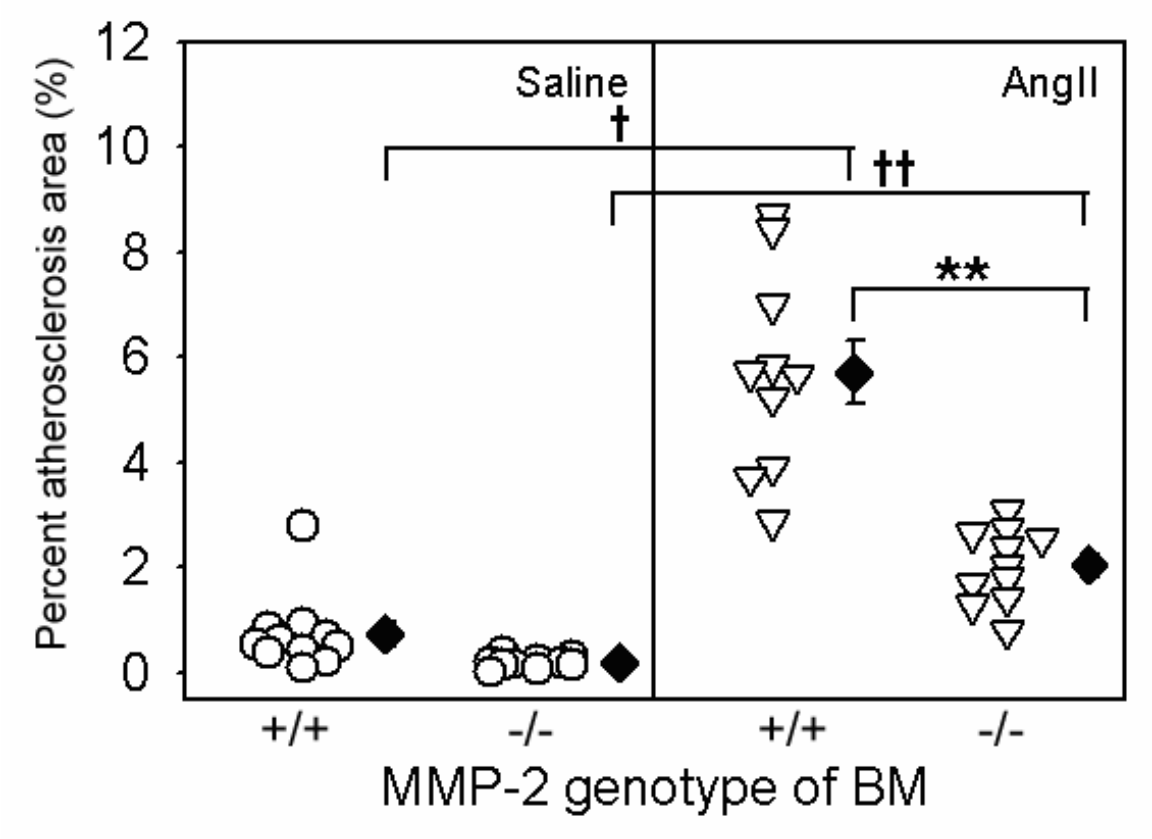


Figure 5.8 Effect of bone marrow-derived cell transplantation on AngII-induced atherosclerosis on the intima of the aortic arch and thoracic aorta of recipient mice*.* The percentage of the intimal area covered by atherosclerotic lesion was quantified in the aortic arch and thoracic aorta. Infusion of AngII increased the area of atherosclerosis in both MMP-2 +/+ and -/- bone marrow-derived cell recipient mice. There was a significant reduction in AngII-induced atherosclerosis in MMP-2-/- BM-derived cells recipient mice compared to MMP-2+/+ BM-derived cells recipient mice (n = 10-11/group; ** $P < 0.001$ for comparisons of MMP-2 -/- versus MMP-2+/+ within AngII infused groups; † $P < 0.001$ for comparisons of AngII versus saline infusion within MMP-2+/+ mice; †† $P < 0.01$ for comparisons of AngII versus saline infusion within MMP-2 -/- mice.

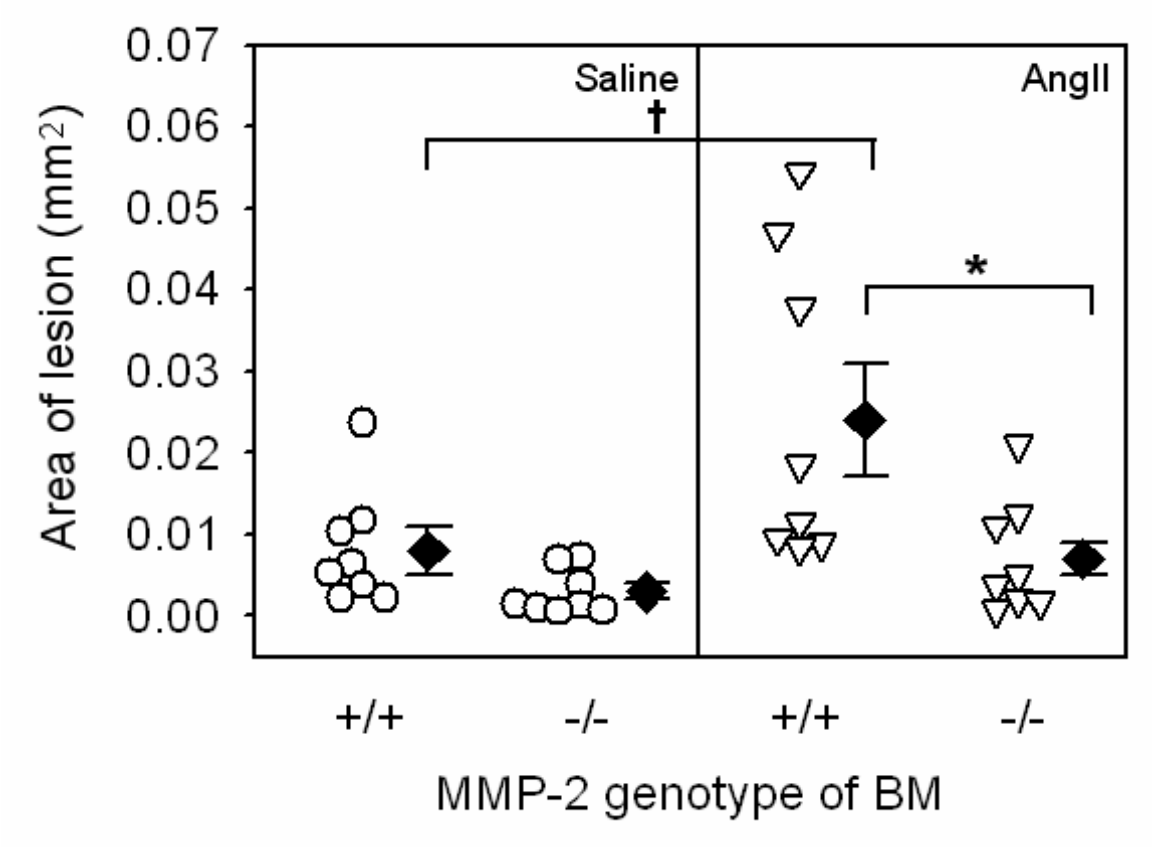


Figure 5.9 Effect of bone marrow-derived cell transplantation on AngII-induced atherosclerosis in aortic roots of recipient mice Reduced severity of atherosclerosis was observed in the sections of aortic root in mice with MMP-2-/- bone marrow-derived cells. Values obtained from individual mice are represented as open symbols, the means are depicted as closed symbols and SEM as bars. (n = 8/group; *P < 0.01 for comparisons of MMP2+/+ versus -/- within AngII or saline infused groups; †P < 0.01 for comparisons of AngII versus saline infusion within MMP-2+/+ mice)

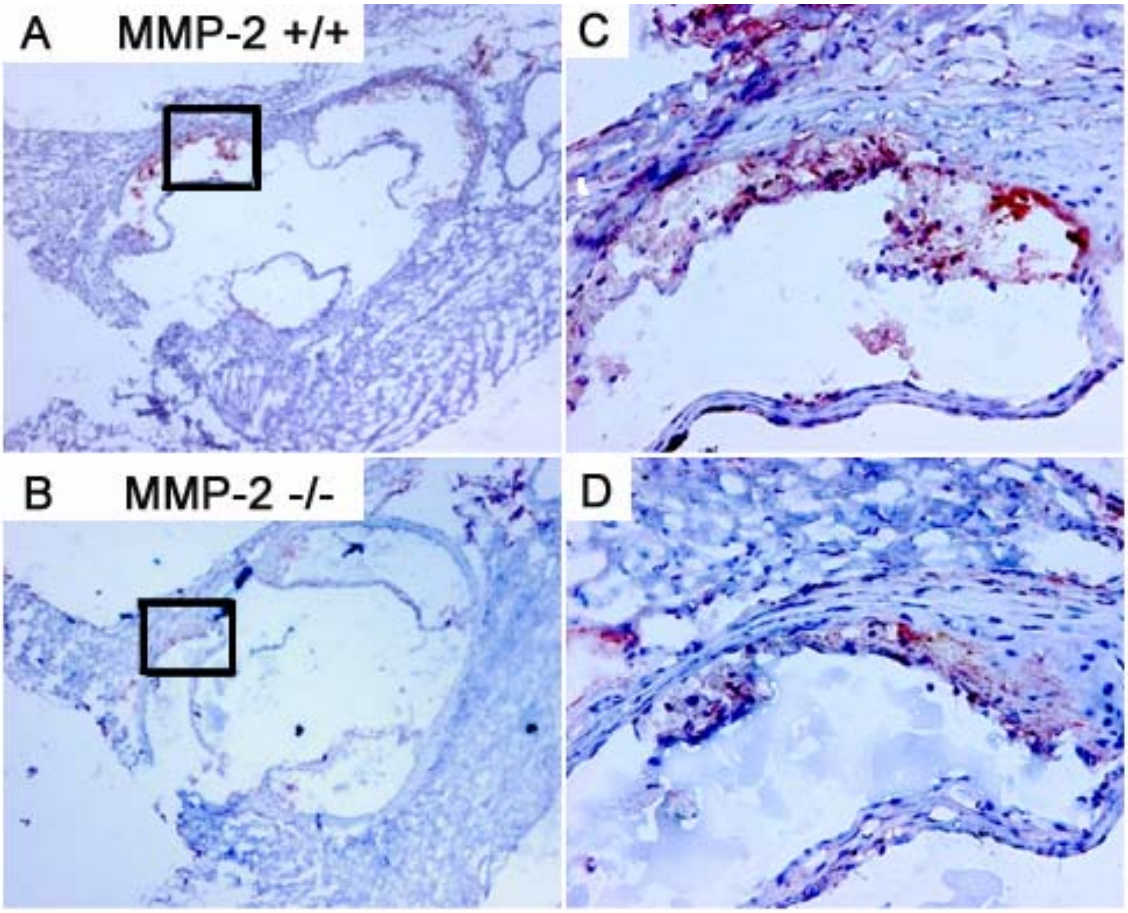


Figure 5.10 Effect of bone marrow-derived cell transplantation on macrophage infiltration in AngII-induced atherosclerosis in aortic roots from recipient mice Representative photomicrographs of immunohistochemical staining for macrophage on cross-cryosections of aortic roots were shown. Staining was less pronounced in MMP-2-/- bone marrow cell recipient mice (B) as compared to MMP-2+/+ bone marrow recipient mice (A). This indicates a reduced macrophage infiltration in the atherosclerotic lesions in MMP-2-/- bone marrow cell recipient mice. However, it might be due to a reduced size of atherosclerosis in these mice. Bars indicate 50 μm. C and D show the detail of boxed area in A and B, respectively.

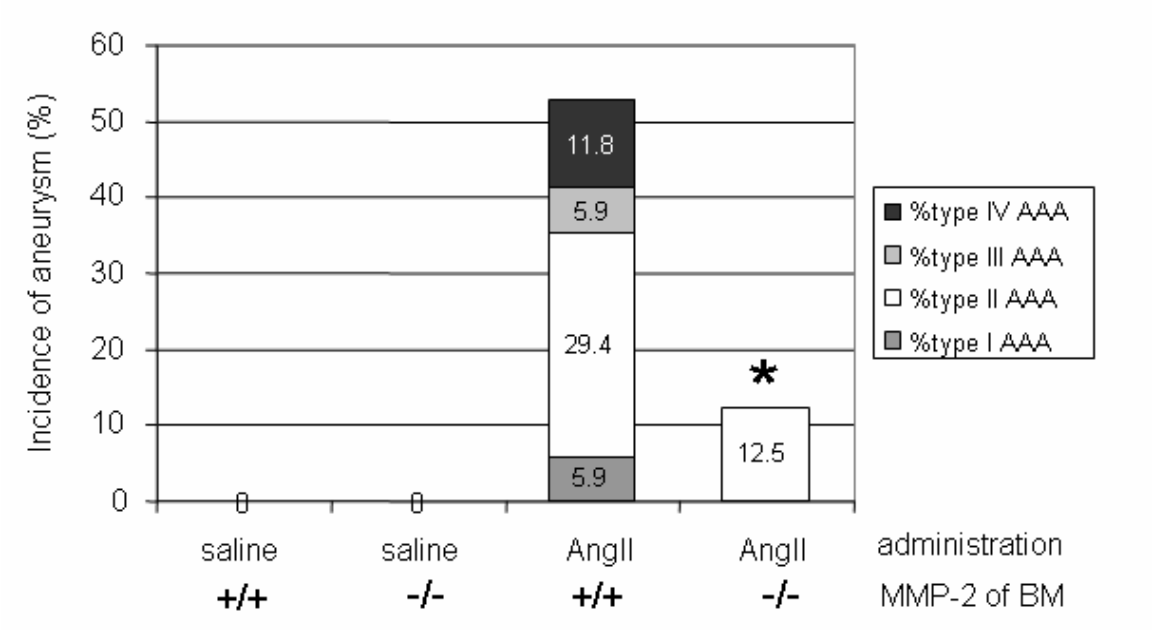


Figure 5.11 Effect of bone marrow-derived cell transplantation on the incidence of AngII-induced AAAs in recipient mice The absence of MMP-2 in bone marrow-derived cells decreased the incidence of AngII-induced AAA in apoE -/- mice compared with MMP-2 wild type bone marrow transplanted mice. (n = 14-17 per group; *$P < 0.05$) BM is for bone marrow.

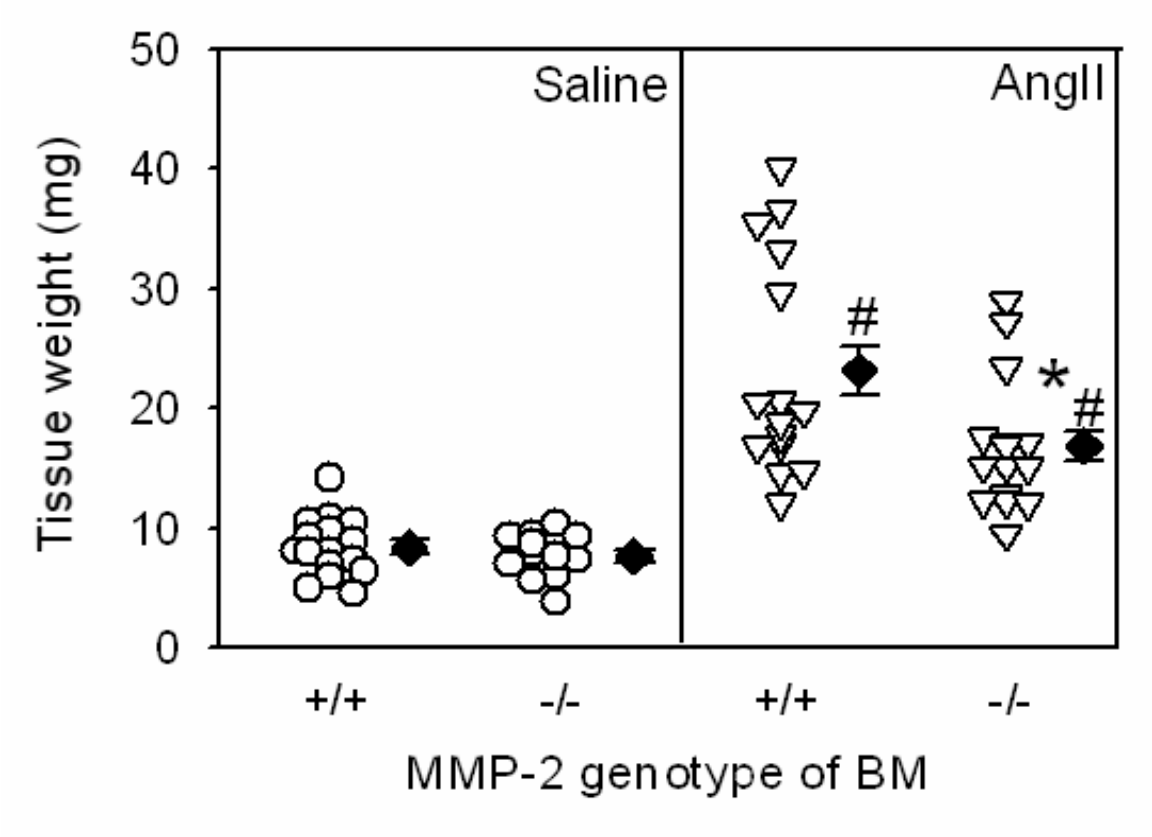


Figure 5.12 Effect of bone marrow-derived cell transplantation on the severity of AngII-induced AAAs in recipient mice Abdominal aortas were isolated by cutting the aorta at the level of the diaphragm and the iliac bifurcation. The tissue was weighed to provide an index of AAA severity. Values obtained from individual mice are represented in open symbols, the means are shown as closed symbols and SEM as bars (n = 14-16 per group). These data do not include the tissue weight of two mice (both were MMP-2+/+ bone marrow cell recipient mice) that died from AAA rupture during AngII infusion. * $P < 0.05$ for comparisons of MMP2+/+ versus -/- within AngII infused mice; #$P < 0.01$ for comparisons of AngII versus saline infusion within mice with same MMP-2 genotypes

Chapter Six

General Discussion and Future Studies

Atherosclerosis and AAAs are distinguishable, vascular diseases that have some common features. For example, they share certain risk factors such as smoking, aging, and male gender. Furthermore, population-based studies demonstrate that a considerable number of patients with AAAs have a history of atherosclerosis. Two prominent pathological characteristics of both atherosclerosis and AAAs are the infiltration of inflammatory cells (especially macrophages and lymphocytes) into the vascular wall and the remodeling of the extracellular matrix (ECM) at the site of inflammation.[63,170] Proteases, especially MMPs, and cytokines play key roles in these pathological processes.

There is evidence that MMP-2 is involved in atherosclerosis and AAA formation. Primarily, the secretion and activation of MMP-2 is elevated in both atherosclerosis and AAA lesions.[43,74] Also, abluminal application of $CaCl_2$-induced AAA formation was ablated in MMP-2 knock out mice.[126] MMP-2 deficiency led to reduced intimal hyperplasia in mouse carotid artery blood flow cessation model as well.[101,114] The understanding of the mechanism behind these results is still superficial, although some hypothesize that MMP-2 functions in ECM remodeling and facilitating cell migration by destroying the barrier around the cell.

The primary purpose of the current dissertation project was to evaluate the general hypothesis that MMP-2 plays an important role in hyperlipidemia and AngII-induced atherosclerosis and AAAs. We also sought to determine if there is a greater role of MMP-2 beyond just the cleavage of elastin. By using apoE-/- double knockout mice in this project, we were able to evaluate the role of MMP-2 deficiency in hyperlipidemia as well as atherosclerosis. Four studies were completed to test the overall hypothesis.

First, we investigated the development of atherosclerosis and AngII-induced AAAs in MMP-2-/- and MMP+/+ mice on an apoE-/- background. It was

surprising that MMP-2 deficiency did not reduce the incidence of AngII-induced AAAs or the size of atherosclerotic lesions in apoE-/- mice. However, the cellular and ECM content of atherosclerotic plaques was modified in MMP-2-/- x apoE-/- when compared to MMP-2+/+ x apoE-/- control mice. To explain the apparent paradox between this result and the hypothesis, we investigated the morphological characteristics of the aortic wall of MMP-2-/- mice in a hyperlipidemic background. We detected an enhanced MMP-9 level in the aortic wall of MMP-2-/- x apoE-/- mice compared with MMP-2+/+ x apoE-/- mice. Interestingly, we also observed more branches in the elastin fibers of the aortic wall from MMP-2-/- mice when compared with aortic walls of wild type mice. Next, we examined the behavior of macrophages of MMP-2-/- mice. Reduced adhesion, migration and expression of integrin beta 3 were detected in the macrophages of MMP-2 deficient mice when compared with the macrophages of wild type control mice. Lastly, to examine whether MMP-2 deficiency in bone marrow-derived cells could influence AAAs and atherosclerosis, we implemented a phase of bone marrow transplantation. There was a significant reduction of both atherosclerosis development and AAAs formation in mice receiving reconstituted MMP-2-/- bone marrow cells.

The most encouraging finding in this dissertation project is that bone marrow cell-derived MMP-2 is critical in AngII-induced AAAs and atherosclerosis in apoE-/- mice. In spite of enhancing MMP-9 expression (mainly in an inactive form), the peritoneal macrophages of MMP-2-/- mice have an impaired adhesion and invasion ability, which might be due to a reduced integrin beta 3 expression. Consistent with this finding, it was reported that a 10-fold increase in the inactive form of MMP-9 in macrophages by gene transfection did not reduce the atherosclerotic lesion in apoE deficient mice (Data presented by P. Gough at the 6th annual conference on Atherosclerosis, Thrombosis and Vascular Biology, 2005). Although MMP-9 can be activated by several serine proteinases, the active form of MMP-2, generated after the activation of TIMP-2-free proMMP-2 on the cell surface, might activate proMMP-9. Based on our findings, we concluded that MMP-2 deficiency in bone marrow-derived cells reduced AngII-

induced AAAs and atherosclerosis, and this reduction might be due to reduced adhesion and migration ability of macrophages. According to the shortcomings of MMP inhibitors, such as toxicity and lack of specificity, these findings could lead to new targeted-molecular approaches to the prevention and treatment of atherosclerosis and AAAs.

At first, it seems that there is a contradiction between the dramatic protective effect from MMP-2 deficiency in bone marrow-derived cells and the data we obtained from the study of MMP-2-/- x apoE-/- mice, in which MMP-2 deficiency did not reduce the incidence of AAAs or the size of atherosclerosis. However, this discrepancy might provide an insight into the importance of the interaction among various cell types and MMPs in the development of vascular diseases. There are two hypotheses which may account for this discrepancy: 1) MMP-2 produced by resident cells of the vessel wall (especially SMCs) is supplemental in atherogenesis and AAA formation and 2) the overexpression of MMP-9 in SMCs is critical for atherosclerosis and AAA formation in apoE-/- mice, but the overexpression of MMP-9 in macrophages is not.

Although inflammatory cells play a pivotal role in the initiation and progression of both diseases, the participation of other cell types, especially VSMCs and endothelial cells, is critical as well. In AAAs, VSMCs play a key role in the remodeling of aortic wall. VSMCs synthesize matrix proteins, such as collagen, elastin, and proteoglycans. They also secrete proteolytic enzymes, including MMPs, that digest the matrix proteins and turnover the ECM of the aortic wall. In rats, implantation of VSMCs prevented AAA formation in an aortic xenograft model.[4] Proliferation and migration of VSMCs in the subendothelial space is a prominent pathological feature of atherosclerotic lesions. Multiple MMPs (including MMP-2 and 9) are involved in the migration of VSMCs. Although MMP-2 and MMP-9 have a redundancy in their substrate profile, it is possible that they bind to a diverse range of receptors and elicit different patterns of cell behaviors in various types of cell. On the basis of our study, we concluded that integrin alpha v beta 3 might serve as a receptor for MMP-2 on the macrophage membrane. This conclusion is supported by our data suggesting

that TSRI265 reduced the invasion ability of MPMs. Enhanced MMP-9 expression did not reconstitute this adhesion ability in MMP-2 deficient macrophages. Although MMP-9 is produced in relatively small amounts by VSMCs when compared to MMP-2, it appears to regulate smooth muscle cell adhesion and migration through its interaction with CD44, a hyaluronan receptor.[101] Taken together, these data highly suggested that MMPs bind to various receptors and influence cell behaviors through autocrine and/or paracrine pathways. However, more studies must be done to elucidate mechanisms underlying the interaction and regulation of these enzymes and their receptors to verify these hypotheses. In MMP-2-/- x apoE-/- mice, the increased MMP-9 expression in the endothelium and/or VSMCs might overwhelm the beneficial effect from MMP-2 deficiency in macrophages.

MMP-2 might influence the proliferation and apoptosis of VSMCs as well. First, the proliferation of VSMCs is elicited by multiple growth factors, such as TGF-beta, IGF, and bFGF, through autocrine and/or paracrine pathways.[12] Although the mechanisms underlying these pathways are not fully understood, it is reasonable to hypothesize that MMP-2 might be involved in the VSMCs proliferation through modifying the activity of growth factors. Second, MMPs might process and release the Fas ligand (FasL), which is an integral membrane protein homologous with TNF and mediates apoptosis of Fas^+ target cells, in a soluble form (sFasL). Treatment with inhibitors of MMPs led to a reduction of sFasL and an accumulation of membrane-type Fas ligand (mFasL).[102,105,198] sFasL, lacking transmembrane and cytoplasmic domains, is a poor mediator of apoptosis, whereas full-length mFasL is pro-apoptotic. sFasL derived from monocytes/macrophages and endothelial cells induces VSMCs apoptosis.[18] This apoptotic effect from deregulation of growth factors and/or ablation of FasL processing might contribute to the diminished protection from AngII-induced AAAs in MMP-2 deficient, hyperlipidemic mice. More studies need to be done to test these hypotheses in future.

As mentioned in Chapter Three, two potential mechanisms might underlie the enhancement of MMP-9 expression in the aortic wall of MMP-2-/- x apoE-/-

mice. First, there might be compensatory overproduction MMP-9 in mice with MMP-2 gene deficiency. Second, it was reported that the MT1-MMP/TIMP-2/MMP-2 complex, in addition to facilitating activation of pro-MMP-2, could be involved in regulating the expression of MMP-9.[53] This indicates that MMP-2 might be a critical component in the regulation of MMP-9 expression.

Both ECM remodeling and inflammatory cell recruitment are important for the maintenance of normal vascular physiological functions, such as healing post-injury. As a constitutively expressed enzyme, MMP-2 is involved in both processes. However, under pathological conditions, these processes might become overactive and shift to being harmful rather than protective for blood vessels, facilitating the development of atherosclerosis and AAAs. Therefore, a temporal or spatial regulation of MMP-2 instead of a simple deletion of the MMP-2 gene might emerge as a desired prophylaxis and treatment of vascular diseases. To reach this goal, a full understanding of the redundant ECM proteolytic mechanisms (i.e. why are so many proteolytic enzymes involved in the remodeling of vascular wall?) in the development of vascular diseases is also critical. This requires the further understanding of the physiopathological specific situation, the cooperation of diverse types of cells, and the stimuli which induce synthesis and/or activation of individual MMPs in vascular diseases as well.

Through careful research and evaluation, the importance of MMP-2 in cardiovascular diseases has been realized in the last decade. Traditionally, MMP-2 was thought to release cells from the ECM simply by its elastiolytic capability. However, the role of MMP-2 in ECM degradation may be more complex. Combined with the realization of the critical role of ECM in regulating signaling molecules for cell behaviors, other insights were taken into the evaluation of the potential role of ECM-digestive enzymes in both physiological and pathological processes. Our major findings in the current dissertation support that the role of MMP-2, which is an important extracellular protease and constitutively expressed in vascular wall, goes far beyond that of remodeling the ECM only. MMP-2 can be involved in the regulation of cell behaviors as well. This new perspective may lead to exciting new approaches in the intervention of

cardiovascular diseases. It could be pivotal in understanding the subtle interaction involved in cell-to-matrix and cell-to-cell interaction. However, future studies will have to determine the pathways required by these enzymes for the activation and regulation of various cell types under diverse physiopathological conditions. These studies might contribute to a further understanding of the role of MMP-2 in vascular diseases.

ABBREVIATIONS

AA	amino acid
AAA	abdominal aortic aneurysm
ACE	angiotensin-converting enzyme
AngII	angiotensin II
apoE	apolipoprotein E
BM	bone marrow
BP	blood pressure
CD	cluster differentiation
CHD	coronary heart disease
ECM	extracellular matrix
EGF	epidermal growth factor
EPC	endothelial progenitor cell
ET	endothelin
FACS	fluorescence activated cell sorting/flow cytometry
FGF	fibroblast growth factor
Hb	hemoglobin
HDL	high density lipoprotein
ICAM	intercellular adhesion molecule
IGF	insulin-like growth factor
IGFBP	insulin-like growth factor binding protein
LDL	low density lipoprotein
IL	interleukin
LRP	low density lipoprotein-related protein
MCP-1	macrophage chemoattractant protein-1
MMP	matrix metalloproteinase
MPM	mouse peritoneal macrophage
MT-MMP	membrane type-MMP
O.C.T.	optimal cutting temperature

Opn	osteopontin
ox-LDL	oxidized LDL
PAI-1	plasminogen activator inhibitor-1
PCAM	platelet endothelial cellular adhesion molecule
PCR	polymerase chain reaction
PDGF	platelet-derived growth factor
PG	prostaglandin
RAAS	renin-angiotensin-aldosterone system
RAP	receptor-associated protein
RBC	red blood cell
ROS	reactive oxygen species
RT	room temperature
RT-PCR	reverse transcription polymerase chain reaction
TC	total cholesterol
TGF	transforming growth factor
TIMP	tissue inhibitor of matrix metalloproteinase
tPA	tissue plasminogen activator
uPA	urokinase-type plasminogen activator
VCAM	vascular cell adhesion molecule
VEGF	vascular endothelial growth factor
VSMC	vascular smooth cell
WBC	white blood cell

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VITA

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PERSONAL INFORMATION

Date of Birth:	November 13th, 1971
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EDUCATION

Jan. 2001 – Present	Graduate Center of Toxicology, University of Kentucky, Lexington, KY Ph.D. Candidate; dissertation pending (expected graduation date June, 2005)
Sep. 1988 – July 1993	Department of Clinical Medicine, Capital University of Medical Sciences, Beijing, China M.D.

WORKING EXPERIENCE

Jan. 2001–Present	Research Assistant, Graduate Center of Toxicology, University of Kentucky, Lexington, KY
Sept.1993–Dec.1999	Department of Cardiology, Yanjing Hospital, Beijing, China Resident (9/1993-7/1997); Superior Resident (7/1997-7/1998); Attending Physician (7/1998-12/1999);

AWARDS/SCHOLARSHIPS

2003-2005 Pre-doc fellowship from American Heart Association ($18,000/year)

2003-2005 Award of graduate student incentive program from University of Kentucky ($900/year)

2003 New Investigator Travel Award from the 4th annual conference on ATVB (Arteriosclerosis, Thrombosis and Vascular Biology; $1,000)

2001-2002 Scholarship from Graduate Center of Toxicology, University of Kentucky ($18,000/year)

1998 Prize for Advance in Science and Technology (Province level, China)

1993 Liu Zhanmou (Professor of Baigang Central Hospital, Japan) Applied Medicine Scholarship

1989-1993 Top Student Prize awarded from Capital University of Medical Sciences

1988-1993 University Scholarship from Capital University of Medical Sciences

MEMBERSHIP

Society of Toxicology (student member)

PUBLICATIONS

1. **Jing Huang**, Lisa A Cassis, Thomas E Curry and Alan Daugherty. Morphological changes and vascular diseases in MMP-2 deficient mice. (in preparation)

2. **Jing Huang**, Lisa A Cassis, Thomas E Curry and Alan Daugherty. MMP-2 deficiency in bone marrow-derived cells reduced AngII-induced abdominal aortic aneurysms and atherosclerosis in aopE deficient mice. (draft finished)

3. Tracy A Henriques, **Jing Huang**, Susan S D'Souza, Alan Daugherty, Lisa A Cassis. Orchiectomy, but not ovariectomy, regulates angiotensin II-induced vascular diseases in apolipoprotein E deficient mice. *Endocrinology* 2004; 145(8): 3866-72.

4. Lisa A Cassis, **Jing Huang**, Ming C Gong, Alan Daugherty. Role of metabolism and receptor responsiveness in the attenuated responses to Angiotensin II in mice compared to rats. *Regul Pept*. 2004;117(2):107-16

5. Michael W. Manning, Lisa A Cassis, **Jing Huang**, Stephen J Szilvassy and Alan Daugherty. Abdominal aortic aneurysms: fresh insights from a novel animal model of the disease. *Vascular Medicine* 2002; 7(1): 45-54

PRESENTATIONS AND PUBLIC APPEARANCES

1. **Jing Huang**, Lisa A Cassis, Thomas E Curry and Alan Daugherty. MMP-2 deficiency leads to marked changes in hemopoetic and vascular cells. Poster Presentation. Southeast Lipid Research Conference. *Sept. 2004*; Pine Mountain, GA.

2. **Jing Huang**, Lisa A Cassis, Stephen J Szilvassy, Thomas E Curry and Alan Daugherty. MMP-2 deficiency decreases the adhesion activity of mouse peritoneal macrophages through reduced integrin alpha v beta 3 expression. Poster Presentation. Abstract P445 5th Annual Conference on Arteriosclerosis, Thrombosis and Vascular Biology *May, 2004;* San Francisco, CA: Society for American Heart Association

3. **Jing Huang,** MMP-2 deficiency in bone marrow derived-cells reduced AngII-induced vascular diseases and the potent mechanisms*. Apr. 2004*; Graduate Center of Toxicology, University of Kentucky, Seminar Series

4. **Jing Huang**, Lisa A Cassis, Thomas E Curry and Alan Daugherty. Failure of MMP-2 Deficiency to influence AngII-induced vascular diseases in apoE-/- mice: potential compensatory role of MMP-9? Poster Presentation No. 29; *Oct. 2003*; Lexington Kentudky: Gill Heart Institute Cardiovascular Research Day

5. **Jing Huang,** Lisa A Cassis, Stephen J Szilvassy, Thomas E Curry and Alan Daugherty. Deficiency of matrix metalloproteinase-2 in bone marrow-derived cells decreases the incidence of Angiotensin II-induced abdominal aortic aneurysm in apolipoprotein E-/- mice. Poster Presentation. Abstract P337; 4th Annual Conference on Arteriosclerosis, Thrombosis and Vascular Biology May, 2003; Washinton, D.C.: Society for American Heart Association

6. **Jing Huang,** Lisa A Cassis, Stephen J Szilvassy, Thomas E Curry and Alan Daugherty. Deficiency of matrix metalloproteinase-2 in bone marrow-derived cells decreases the incidence of Angiotensin II-induced abdominal aortic aneurysm in apolipoprotein E-/- mice. Poster Presentation No. 10; Nov. 2002; Lexington Kentucky: Gill Heart Institute Cardiovascular Research Day

7. **Jing Huang,** Lisa A Cassis, Alan Daugherty. Hypertension induced by AngII infusion in C57BL/6 mice. Poster Presentation No. 54; Oct. 2001; Lexington Kentucky: Gill Heart Institute Cardiovascular Research Day